Anti-hypertensive effect of Korean fermented soybean paste (Doenjang) through regulation of the renin-angiotensin system (RAS) in male rats fed with high-fat and/or high-salt diet

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Research

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Abstract

Background: Korean fermented food, *doenjang*, is questioned due to its high salt content, although it has been reported an abundance of beneficial effects. Therefore, we investigated its impact on renin-angiotensin system (RAS) hypertension using 3T3-L1 adipocytes and male Sprague-Dawley rats.

Results: Among the rats fed with normal diet (ND), high-fat diet (HD), high-fat diet with 8% table salt (HDS), or high-fat diet with *doenjang* containing 8% table salt (HDJ) for 13 weeks, the HDJ group showed significantly lower blood pressure, lesser body weight, and reduced levels of serum angiotensin II and aldosterone, compared to the HD and HDS groups. In addition, mRNA expressions levels of angiotensinogen, angiotensin converting enzyme, angiotensin II receptor type 1, and angiotensin II receptor type 2 were downregulated in epididymal fat of the HDJ group.

Conclusions: In spite of its high salt content, *Doenjang* appears to inhibit obesity-induced hypertension through modulation of RAS by blocking the angiotensin converting enzyme, even with high fat intake.

Background

The incidence of obesity is increasing due to the imbalance between energy intake and consumption, and hence obesity is becoming a global health burden [1]. The prevalence of obesity and obesity-related diseases is increasing worldwide [2]. It is one of the major risk factors for hypertension [3]. Weight gain is related to increased blood pressure; about 60-70% of hypertensive adults excess adiposity [2]. According to WHO, hypertension is estimated to afflict 1.13 billion people worldwide. In 2015, one in five women and one in four men were predisposed to hypertension [4]. Indeed, it is more difficult to regulate blood pressure in obese patients than in ordinary people, and obesity is recognized as a common cause of treatment-resistant hypertension [5].

The Renin-angiotensin system (RAS) is well known for involved in controlling blood pressure and fluid/electrolyte balance. Activated RAS is a common aspect of metabolic syndrome patients [6]. Angiotensinogen (*Agt*) is the precursor of the bioactive angiotensin peptides, angiotensin I (*Ang I*) and angiotensin II (*Ang II*), produced through two enzyme cleavages, which are renin and angiotensin-converting enzymes (*Ace*), respectively [7]. RAS regulates blood vessel constriction, and reabsorption of Na^+ and water [8]. Recent studies have reported that RAS affects obesity and insulin resistance. Adipose tissue is described as an endocrine organ, which plays a significant role in energy metabolism, and contains all RAS components [9].

Korean traditional soybean paste, called *doenjang*, is known to have antiobesity effects [10]. *Doenjang* is one of the representative products of South Korea. It is prepared using brine and fermented *Meju* and mixed with salt (Fig. 1). The enzyme secreted by Bacillus breaks down the soybean protein and produces an amino acid that creates a savory taste [11]. *Doenjang* is rich in flavonoids, vitamins, minerals, and phytoestrogens, which are the constituents of soybean. Soybeans also contain isoflavones, which have antioxidant, anticancer, anti-inflammatory effects, and can prevent coronary heart diseases [12,13]. Several

in vitro and *in vivo* studies have shown the health benefits of *doenjang* and its active compounds. Our previous clinical studies have reported its antiobesity, antioxidant effects, and its beneficial effects on overall health index. However, consumption of *doenjang* is still questioned, because of its high salt content [12]. Salt is added during the manufacturing of *doenjang* to control the harmful microbial growth and improve its preservation. It promotes the growth of beneficial microbe, inhibits undesirable fermentation, and improves its taste. [11]. However, excessive sodium intake is generally known to increase the risk of hypertension through hypervolemia, promote heart and kidney disease, and contribute to stroke and gastric cancer [14, 15].

In our previous study, we had investigated how *doenjang* regulates hypertension caused by a high-salt diet. *Doenjang*, when fed along with normal diet, caused a significant reduction in the blood pressure and lowered the expression of sodium transferase-related genes, as compared to the same amount of salt alone. [16,17].

In the present study, the antiobesity and antihypertensive effects of traditional fermented foods in the obese condition were studied in various ways. Here, we considered the salt content, which is different from the previous research, which focused on the effects of doenjang effects in normal conditions. We included high-fat as well as high-salt diets in our experiments, which have investigated few other studies, and the results were inconclusive yet [18]. High-salt and high-fat diets synergistically promote obesity and high blood pressure [3]. Purpose of this study was to examine the effect of a combination of high-salt and high-fat diet on RAS in adipocytes and how *doenjang* containing high amount of salt, regulates obesity and blood pressure, even when consumed together with a high-fat diet.

Materials And Methods

Preparation of *Doenjang*

Doenjang was produced by the Sunchang Sauce Corporation of South Korea (Sunchang-gun, Jeollabuk-do, Korea). This fermented food is prepared using soybean after maturing it for 6 months, by traditional Korean fermentation process (Fig. 1). After steamed soybeans, it was made into blocks, and fermented with *Aspergillus oryzae* and *Bacillus subtilis* for one month. After this, it was mixed with brine (saltwater, 26%, w/v) in a 1:3 ratio and further fermented for 2 more months. Once matured, *doenjang* was dried using a freeze-dryer (FD12008, Ilshin Biobase, Gyeonggi-do, Korea), and its salinity was adjusted with NaCl (Samchun, Pyeongtaek-si, Gyeonggi-do, Korea) to 8%, using Mohr's method.

Animals and treatments

Three-week-old male Sprague-Dawley rats were purchased from Central Lab. Animal, Inc. (Seoul, Korea), and acclimated at 12 h light and 12 h dark cycles at 25 ± 2°C and humidity 50% ± 5% conditions. After 7 days adaptation, they were randomly (no diffrence in body weight and initial SBP) divided into 4 groups (n=6); normal diet control (ND; AIN76A, Research Diets, Inc. New Brunswick, NJ, USA), high-fat diet control (HD; 60% fat by weight, D12492, Research Diets, New Brunswick, NJ, USA), high-fat diet with 8% table salt (HDS), and a high-fat diet with *doenjang* containing 8% table salt (HDJ). All animals were fed with their

corresponding experimental diets (Table 1) for 13 weeks, with water supplied. Body weights were noted once a week and food intake was measured each day. Systolic blood pressure (SBP) was recorded weekly by the indirect tail-cuff method (BP-2000, Visitech Systems, Inc., Apex, NC, USA) 30 min after placing them at 37°C. The mean SBP was recorded after 7 measurements. During the last 4 weeks, the animals were individually housed in metabolic cages 24 h for 3 consecutive days in a week, with water provided through drinking bottles and food in the cage. Food and water intake, and fecal and urine output were measured and collected for analyses. Mean value of each criterion was recorded for each rat. All animal procedures were approved by the Animal and Use Committee of Chonbuk National University (CBNU 2018-052).

Tissue collection

At the end of 13 weeks, rats were fasted for 12 h before anesthetizing with 2 mg/kg BW alfaxan (Jurox, Australia) and 0.5 mL/kg BW rompun (Bayer, Seoul, Korea) through intramuscular injection to collect blood and tissues. Liver, epididymal adipose tissue, and one of the kidneys were rinsed with saline, weighed, and immediately frozen in liquid nitrogen and stored at -80°C for further analyses. The other kidney was fixed in 10 % formaldehyde and embedded in paraffin. Blood was drawn by orbital vein puncture and centrifuged at 3,000 rpm for 15 min at 4°C to collect serum.

Biochemical analysis

The serum levels of renin, angiotensin II and aldosterone were measured using assay kits (Rat Renin ELISA Kit, MyBioSource, San Diego, CA, USA; Angiotensin II ELISA Kit, and Aldosterone ELISA Kit, Enzo Life Sciences, Inc., Farmingdale, NY, USA), following the manufacturer's protocols.

Histology of fat cryosections

Frozen epididymal adipose tissues were cryopreserved in OCT (Scigen Scientific Gardena, CA, USA), and frozen in liquid nitrogen. Sections of 10 μm thickness were cut with cryomicrotome (Shandon Cryotome FE, Thermo Scientific, MA, USA), transferred onto glass slide (Marienfeld, Germany) at -30°C. Sections were stained with hematoxylin and eosin (H&E) and mounted in glycerol gelatin. Cells were observed using an Axiophot Zeiss Z1 microscope (Carl Zeiss, Gottingen, Germany) at X200 magnification, and adipocytes were counted. Difference in cell size in each group were noted.

RNA extraction and real-time PCR

Total RNA was isolated from after homogenizing the tissues in TRIzol reagent (Invitrogen, Grand Island, NY, USA), and the concentration of total RNA was equalized using quantifying on Biodrop Duo (Biochrom, Holliston, MA, USA). cDNA was synthesized using PrimeScript™ RT Master Mix (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. RNA expression was measured by quantitative real-time polymerase chain reaction (qPCR) using the SYBR Green real-time PCR master mix (TOYOBO, Osaka, Japan), on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), and quantitative analysis of PCR data were calculated through $2^{-\Delta\Delta Ct}$ method, using beta-actin as an internal control. Primers used for the qPCR are listed in Table 2.

Urine and feces analyses

Urine and feces samples, collected from metabolic cages, were analyzed for their Na^+ and K^+ contents, by inductively coupled plasma-mass spectroscopy (ICP-MS; 7500A, Agilent Technologies, Germantown, MD, USA), the center for University-Wide Research Facilities (CURF) at Jeonbuk National University.

Analyses of serum ion levels

The Na^+ and K^+ concentrations in the serum were determined using Fuji Dri-Chem Slide Na-K-Cl (FUJIFILM, Tokyo, Japan) with FDC 3500i chemistry analyzer (Fuji Dri-Chem Analyzer, Tokyo, Japan).

Preparation of *doenjang* samples for treating the cell line

Doenjang was purchased from the Sunchang Sauce Corporation of South Korea (Sunchang-gun, Jeollabuk-do, Korea). The 1 gram of each freeze-dried *doenjang* was mixed with 10 mL of solvent (80% ethanol) at RT for 24 h on a shaker. The supernatants were collected and filtered through ADVANTEC No. 2 filter paper, and 1 mL of each filtrate was freeze-dried in a speed vacuum concentrator (FD12008, Ilshin Biobase, Gyeonggi-do, Korea).

Study design and cell culture

The 3T3-L1 preadipocyte cell line ((CL-173)-ATCC, VA, USA) was maintained in DMEM (Hyclone, USA) containing 10% bovine serum (Gibco, NY, USA) and 100 U/mL 1% penicillin-streptomycin (Hyclone, USA) at 37°C under 5% CO_2 in a humidified incubator. RNA was extracted from 3T3-L1 cells at different times after differentiation. In order to observe the effect of RAS blockers, the 3T3-L1 cells were seeded in 6-well plates and upon reaching 100% confluence (day 0), they were continued in culture for 48 h. Then the growth medium was replaced with differentiation medium, containing DMEM, 10% fetal bovine serum (FBS), 0.5 µM isobutylmethylxanthine (IBMX), 1 µM dexamethasone (DEXA), and 10 µg/mL insulin (Sigma-Aldrich Co., St. Louis, MO, USA) with or without Losartan (10^{-4} M), Captopril (10^{-4} M), or *doenjang* (0.4% salinity). All treatments chemicals were dissolved in 30% EtOH to match the stock concentrations (Fig. 7). The media, with or without the treatment chemicals, were changed every day, and the cells were harvested on the day 4. Total RNA was extracted using TRIzol reagent, according to the manufacturer's instructions (Invitrogen) and quantified by quantitative real-time PCR with gene-specific primers (Table 3).

Statistical analyses

Results are expressed as mean ± standard error of means (SEM). Statistical analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). One-way ANOVA and Duncan's multiple range test were used for comparison involving three or more groups, and $p < 0.05$ was considered statistically significant.

Results

Body weight, diet intake, and blood glucose level

The initial body weight of the animals did not vary significantly among different groups. After 13 weeks on treatment diets, mean body weight of the HD group rats had significantly increased, as compared to those from HDS and HDJ groups, while the ND group had the lowest body weight (Fig. 2).The group treated with *doenjang* with high-salt, displayed a protective effect against high-fat induced obesity. Diet intake among different groups fed normal diet (ND), and high-fat diet (HD, HDS, HDJ) was not significantly different, respectively (Table 3). Compared to the ND group, the blood glucose levels were elevated in the HD and HDS groups, whereas *doenjang* supplementation markedly attenuated this high-fat diet induced increase in blood glucose (Table 3).

Adipocyte morphology

Animals from the HD and HDS groups showed a significantly higher weight of epididymal fat, compared to HDJ and ND groups (Fig. 3a). However, *doenjang* treated rats from the high-fat diet group showed significantly reduced epididymal fat mass, as well as reduced size of adipocytes in it (Fig. 3b). The ratio of the number of adipocytes to area, was significantly increased in the HDJ group as shown in the histological analysis (Fig. 3c).

Expression of obesity related genes

In order to study the effects of *doenjang* on obesity, the expression of genes related to lipid-regulating enzymes and transcription factors were quantified in the epididymal adipose tissue. *Doenjang* treated rats showed significantly lower expression of leptin mRNA in the epididymal adipose tissue. In contrast, adiponectin expression was upregulated due to *doenjang* supplementation (Fig. 4).

Systolic blood pressure

The base-line SBP in all groups was 110±10 mm Hg, which was significantly increased with high-salt intake over the experimental period, in spite of weight loss. However, SBP of the HDJ group was comparable to that of ND, even though the HDJ diet had the same amount of salt as HDS, indicating that the high-salt content in *doenjang* did not alter the SBP, unlike HDS. Animals from HDJ and ND groups had steady and normal SBP compared to those from HD group also, HDS group showed significantly higher SBP in the last week of the treatment, and it appeared to have reached a plateau at a high of 169.31 mm Hg (Fig. 5).

Renin, angiotensin II, and aldosterone levels in serum

Serum renin level was the highest in the ND group, while the high-fat diet groups showed slightly lower level compared to ND group, still the difference was not significant, whereas only HDS group had significantly less serum rennin than ND group (Fig. 6). Although serum angiotensin II did not significantly decrease in the HDS group compared to the HDJ group, it showed at reducing trend in the latter (Fig. 6). Serum aldosterone also decreased significantly in HDJ (Fig. 6).

Expression of RAS-related genes in adipose tissue

Expression levels of RAS related genes in adipose tissue, angiotensinogen (*Agt*), *renin*, and angiotensin-converting enzyme (*Ace*) mRNA were notably downregulated in HDJ group, whereas they were comparable in HD and HDS groups (Fig. 23-25). Furthermore, animals from the *doenjang* group showed reduced expression of lipogenic genes, including angiotensin II receptor type 1 (*Agtr1*) and angiotensin II receptor type 2 (*Agtr2*), as compared to those from the high salt group (Fig. 7).

Expression of RAS-related genes in kidney and liver

Doenjang treatment reduced *Agt* expression in the liver (Fig. 8), and *Agtr1* in the kidney (Fig. 8), which indicated vasodilation of blood vessels, whereas *Ace* and *Agtr2* in the kidney did not change significantly (Fig. 8). mRNA expression of kidney renin, a feedback controller of angiotensinogen, was upregulated in the *doenjang*-fed group compared to the high-fat, and high-salt groups (Fig. 8). Aldosterone-related genes in kidney, including *Star*, *Hsd3b1*, *Cyp11a1*, *Cyp21*, and *MR*, were downregulated by *doenjang*. In particular, transcription of *Cyp21* and *MR* was drastically reduced in the *doenjang* group (Fig. 9a). Level of renalase deficiency of which increases the blood pressure, was suppressed in both HD and HDS groups, while in HDJ group, it was enhanced (Fig. 9b).

Serum Na^+ / K^+ levels and their excretion

After 24 h in metabolic cages, animals from the HDS and HDJ groups showed a greater amount of water intake and urine excretion than those from the ND and HD groups (Fig. 10). Concentrations of sodium and potassium in both urine and feces were significantly higher in the HDJ group than in the HDS group. However, the ratio of Na^+/K^+ was the highest in the latter.

Serum analysis showed that HD group had the highest concentration of sodium in their serum. In the HDJ group serum sodium levels showed decreasing tendency among the high-fat diet groups, while the serum potassium level decreased significantly in the HDJ group unlike in the HDS group (Table 4).

Effects of *Doenjang* extract on 3T3-L1 cell line

As shown in Fig. 11, treatment with only *doenjang* could lower the key adipogenic transcription factor, *Pparg*, significantly in the differentiated adipocyte after 4 days of exposure, and this was similar to the effects of losartan and captopril. Expression of *Agt* also reduced due to *doenjang* and captopril over the levels seen in the control. Similarly *Ace* gene expression decreased significantly due to treatment with *doenjang* as in the positive control group, treated with Captopril, an *Ace* inhibitor (Fig. 11).

Discussion

Both high-salt and high-fat diets are closely linked and synergistically promote obesity and hypertension. Consumption of high-fat is associated with abnormal accumulation of fat, resulting in an increase in the adipocyte size and/or number [19]. High intake of salt induces hypertension, and extracellular fluid accumulation when the osmolality increases in the extracellular space, due to high Na^+ concentration [20]. Previous studies have shown that *doenjang* inhibits lipid accumulation in the adipocytes. High sodium

content also has been reported to suppress the weight gain with the intake of high-fat diet [21]. Present study showed that *doenjang* and high-salt, lowered the body weight to a level comparable to that of the normal diet fed animals (Fig. 2). Salt affects the activity of renin and angiotensin, and controlled digestive efficiency, and reduces the ability of the gastrointestinal tract to extract calories from the food [21]. This appears to be the underlying mechanism of the body weight reduction in the HDS group. However, bodyweight and obesity-related biomarkers in the HDS group showed an inverse relationship. Adipocyte hypertrophy and hyperplasia were observed in the HD and HDS groups. Interestingly, in the HDJ group had significantly decreased adipocyte size, epididymal fat weight; simultaneously it significantly increased the number of adipocytes to area ratio compared to the HD and HDS group (Fig. 3).

Doenjang, which has soybean as its main ingredient, contains different soy proteins, soy saponin, phospholipids, and isoflavones. These components in soybeans are responsible for its hypolipidemic and antiobesity effects, through increasing *β*-oxidation and inhibiting fatty acid synthesis [10,22]. Our previous study showed that *doenjang* has been lowered bodyweight and improved obesity-related parameters such as insulin, leptin, *Pparg*, and *Acc* [15]. The results of the present study also confirmed the antiobesity effect of *doenjang* evident by the decreased gene expression of leptin, while increased adiponectin levels (Fig. 4). Chronic intake of high-salt diet may increase the risk of renal injury, kidney dysfunction, and hypertension in normal rats [23]. Interestingly, in this study, blood pressure was significantly different in the HDS and HDJ groups, in spite of similar body weights and the same amount of salt intake (Fig. 5). The biopeptides present in *doenjang* may be responsible for its action on RAS, reducing the blood pressure in HDJ group [24].

Obesity is known to induce greater sensitivity towards the changes in blood pressure. Our previous study had indicated that the Korean traditional fermented soybean foods, *doenjang* and ganjang, reduce the blood pressure in normal SD rats [16,17]. RAS is a critical homeostatic regulator that relies on a feedback regulation to achieve and sustain the delicate balance in the blood pressure, required for healthy physiological function [25]. It is activated in the adipose tissue in diet-induced obesity. Expression of systemic RAS is also upregulated in obesity [26]. Therefore, we fed the rats with a high-fat diet to induce obesity and treated them with *doenjang* to study the effects of *doenjang* on RAS expression in adipocytes. Our results showed that expression of adipocyte RAS related genes was significantly downregulated by *doenjang* compared to the salt group. Expression of *Agt*, the first component of the adipocyte RAS, is positively correlated with adipose mass. *Renin*, *Ace*, and *Agtr1* genes are also upregulated in adipose tissue in obesity [27,28]. Ang II promotes lipogenesis by activating *Agtr2*, and simultaneously, by decreasing lipolysis by activating *Agtr1* [29]. The results of the current study also confirmed that *doenjang* reduced lipid accumulation through *Agtr1* and *Agtr2*, leading to suppression of obesity. Adipose-derived angiotensin is also released into the systemic circulation, which regulates the activation of bioactive Ang II, leading to increased levels of plasma Ang II, ultimately resulting in elevated blood pressure. Adipocyte RAS contributes to control of fat mass and can affect systemic functions such as metabolism [27,30]. Increased excretion of *Agt* from fat tissue into the plasma may elevate plasma *Agt*, resulting in increased blood pressure in the obese [6,30]. Our results indicated that adipocyte *Agt* had a pattern similar to that of

plasma angiotensin, which was derived from the liver, and we found that the hepatic *Agt* was also elevated (Fig. 7).

In this study, we evaluated serum levels of RAS and its mRNA expression in kidney. Serum level of renin in the ND group was higher than that in the HDS and HDJ groups, despite the low blood pressure. This is because, in response to the low sodium level in blood, renin is activated [32]. Lower renin levels may be due to abnormal reabsorption of excess renal sodium, decreased renin secretion, and genetic abnormalities in RAS or related genes. High renin hypertension, as well as low renin hypertension appear to be linked with end-stage organ damage, and worsen the prognosis of hypertension than the hypertension with normal renin levels [33]. High concentration of renin is not always associated with high enzyme activity, but it is directly proportional to renin mRNA expression, renin secretion, and plasma renin activity [16,34]. A previous study has also shown that renin mRNA expression tended to be suppressed by the high-salt diet [35]. It appears that renal renin may affect serum renin. Non-proteolytically enhanced catalyzing activity of renin or pro-renin, when bound to pro-renin receptor (PRR), elevates angiotensin I production from angiotensinogen, thereby enhancing RAS [36]. Reduced renin can be explained through a negative feedback loop involving Ang II, which regulates expression and secretion of renin [37]. The present study showed that serum Ang II and renal *Ace* were higher in the high-salt group as a result of upregulated Ang II, leading to the downregulation of renin through the negative feedback loop. The patterns of *Ace* gene expression are similar to systemic *Ace* activity [8]. Systemic *Ace* is the main enzyme responsible for the production of Ang II from Ang I in the intravascular space [38]. Ang II enhances hypertension by increasing oxidative stress and activating angiotensin II receptors [39]. In this study, the levels of *Agtr1* in the kidney showed a decreasing trend in the HDJ group, while *Agtr2* increased significantly in the *doenjang* group, compared to the high-salt group (Fig. 8). *Agtr1* is distributed throughout the kidney and contributes to most of the Ang II actions [40]. Ang II activates *Agtr1* to bring about a variety of biological responses, such as vasoconstriction, renal sodium reabsorption, cell proliferation, cell dedifferentiation, and growth, and increased aldosterone secretion, which contribute to increased blood pressure and the development of hypertension [41]. Actions mediated through *Agtr2* generally counterbalance the actions of *Agtr1* [40]. Aldosterone levels in serum, catalyzed by Ang II, were significantly decreased in the ND and HDJ groups, compared to the levels in HD and HDS groups in the present study (Fig. 6). Similar trends were also observed in aldosterone-related mRNA expression in the kidney. Expression of *Star, Hsd3b1*, *Cyp11a1, Cyp21,* and *MR* genes decreased in the HDJ group (Fig. 9a). In particular, *doenjang* reduced the expression of *Cyp21* and *MR* genes significantly. Aldosterone is synthesized by the action of *Cyp21* in the adrenal cortex [42]. Mineralocorticoid receptor (*MR*), a ligand-dependent transcription factor, mediates the actions of aldosterone in different tissues [43]. Ang II causes vasoconstriction and activates aldosterone, a mineralocorticoid that reabsorbs sodium in the distal tubules and collecting ducts of the kidney at the expense of potassium excretion. This increased vasoconstriction and sodium reabsorption contribute to higher blood pressure and fluid retention [44]. In accordance with this mechanism, our study showed that serum Na^+ levels were only marginally higher in the HDS group than in the HDJ group, but no significant difference was observed, whereas serum K^+ levels were significantly higher in the HD and HDS groups (Table 4). Release of aldosterone from the adrenal cortex is enhanced by high serum concentration of potassium, regardless of the action of Ang II [40]. Consumption of high-salt diets increases the frequency

of urination, because of the stimulated thirst, and excessive water intake [45]. Our study also demonstrated that water intake and urine output increased significantly in the high-salt and *doenjang* groups due to 8% of salt was contained in the HDS and HDJ diets (Fig. 10).

Potassium excretion in urine and feces was significantly lower in the HDS group than in the HDJ group in the present study, while sodium excretion was not noticeably different. Moreover, the Na^+/K^+ ratio was the highest in the HDS group (Table 4). This ratio in urine may contribute to the deterioration of renal function. It was found that the higher the sodium excretion through urine, the lower the potassium excretion, which means that the elevated urine Na^+/K^+ ratio is associated with increased blood pressure, leading to faster deterioration of renal function [46]. Kidney RAS is essential for Na^+ excretion, and for controlling blood pressure [41]. It can also act independent of plasma RAS level upon high-salt intake. Aberrant activation of the kidney RAS generates several factors responsible for hypertension, regulated by a variety of pathophysiological mechanisms related to hypertension and kidney damage [17,39]. The kidney RAS was found and identified to be possibly related to modulation of renalase enzyme. Increased dietary sodium intake decreases the renalase levels in kidney and plasma, and significantly increases the expression of *Agtr1* in kidney, a critical element of kidney RAS [47]. Similarly, in the present study, high-salt intake induced kidney RAS, and it reduced the expression of renalase gene in the renal tissue (Fig. 9b).

Exact mechanism of action and the target of *doenjang* were determined using 3T3-L1 cell line, and compared them with those of Losartan and Captopril, which are antihypertensive drugs that inhibit *Agtr1* and *Ace*, respectively, in the adipocytes. *Pparg*, *Agt*, and *Ace* reduced significantly in the 3T3-L1 adipocytes treated with *doenjang* (Fig. 11), indicating that fat production was inhibited by *doenjang*. Therefore, all these data revealed that *doenjang* regulates blood pressure through RAS, by inhibiting *Ace* in adipocytes.

Conclusion

Results of this study revealed that *doenjang* exerts its beneficial effects by reducing body weight, white adipose tissue, and lipid contents of serum and liver, and improves the obesity-induced high blood pressure. *Doenjang* supplementation downregulated the expression of genes related to adipocyte RAS, leading to the regulation of systemic RAS. This, in turn, affects the kidney RAS, which ameliorates kidney dysfunction and metabolic syndrome. In the doenjang treated rats, serum ions and excretion of Na^+ and K^+ ions differ drastically from those treated with high salt. To sum up, it is clear that *doenjang* ameliorates hypertension by suppressing the elevated blood pressure, even though it contains high-salt. Our data suggests that *doenjang* may inhibit *Ace* in adipocytes, through a mechanism similar to that of Captopril.

Abbreviations

Ace: Angiotensin-converting enzymes

Agt: Angiotensinogen

Agtr1: Angiotensin II receptor type 1

Agtr2: Angiotensin II receptor type 2

Ang I: Angiotensin I

Ang II: Angiotensin II

Cyp11a1: cholesterol side-chain cleavage enzyme

Cyp21: 21-hydroxylase genes

Hsd3b1: 3β-hydroxysteroid dehydrogenase type 1

MR: Mineralocorticoid receptor

Pparγ: Peroxisome proliferator-activated receptor gamma

RAS: Renin-angiotensin system

SBP: Systolic blood pressure

Star: Steroidogenic acute regulatory protein

Declarations

Ethics approval and consent to participate: All animal procedures were approved by the Animal and Use Committee of Chonbuk National University (CBNU 2018-052).

Consent for publication: Not applicable

Availability of data and materials: All of the data are available with reasonable request from the corresponding author.

Competing interests: No potential conflict of interest was reported by the authors.

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Authors' Contributions: HW performed the experiments drafted the manuscript. JEP designed the study and interpreted the results. YSC was the principal investigator.

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Tables

Table 1 The composition of experimental diets

Ingredient	ND	HD	HDS	HDJ
Casein	20	25.85	23.96	22.93
L-Cystine	0.3	0.39	0.36	0.34
Corn Starch	15	0	0	0
Maltodextrin		16.15	14.97	14.33
Sucrose	50	8.89	8.24	7.89
Cellulose	5	6.46	5.99	5.73
Corn/Soybean Oil	5	3.23	2.99	2.87
Lard		31.66	29.35	28.08
Mineral Mix	3.5	1.29	1.20	1.15
DiCalcium Phosphate		1.68	1.56	1.49
Calcium Carbonate		0.71	0.66	0.63
Potassium Citrate		2.13	1.98	1.89
Vitamin Mix	1	1.29	1.20	1.15
Choline Bitartrate	0.2	0.26	0.24	0.23
NaCl (Table salt)			7.3	6.3
Dried *doenjang*				5
total	100	100	100	100
kcal/g	3.9	5.24	4.86	4.82

ND; AIN76A, Research Diets, Inc. New Brunswick, NJ, USA), (HD; 60% kcal% fat, D12492, Research Diets, New Brunswick, NJ, USA), (HDS; HD + 8% table salt), (HDJ; HD + *Doenjang* containing 8% table salt

Table 2 Sequences of primers used for PCR

Gene	Forward	Reverse
Pparγ	ACCACTCGCATTCCTTTGAC	CCACAGACTCGGCACTCAAT
Leptin	TGACACCAAAACCCTCATCA	TCATTGGCTATCTGCAGCAC
Adiponectin	GCACTGGCAAGTTCTACTGCAA	GTAGGTGAAGAGAACGGCCTTGT
Agt	GATGCGCACAAGGTCCTG	CAGGGTGCTGTCCACACTGGCTCGC
renin	TTCTCTCCCAGAGGGTGCTA	CCCTCCTCACACAACAAGGT
Ace	GAGCCATCCTTCCCTTTTTC	GGCTGCAGCTCCTGGTATAG
Agtr1	ACTCTTTCCTACCGCCCTTC	TTAGCCCAAATGGTCCTCTG
Agtr2	GAAGGACAACTTCAGTTTTGC	CAAGGGGAACTACATAAGATGC
Star	GACCAGCCCATGGACAGACTC	AGGTCAATAGTGAGCAGCCA
Hsd3b1	ATGCCCAGTACCTGAGGAGA	TTGAGGGCCGCAAGTATCA
Cyp11a1	AGAAGCTGGGCAACATGGAGTCAG	TCACATCCCAGGCAGCTGCATGGT
Cyp21	CATCGTGCAACTAAGGCTAG	TGGAAGGGAGGAATTAAGAG
MR	GCTTTGATGGTAGCTGCG	TGAGCACCAATCCGGTAG
Renalase	TGACCTTGTCATCCTCACCA	AACTCCAAATGGGACAGTGG
ß-actin	AGCCTTCCTTCTTGGGTATGG	CACTTGCGGTGCACGGTATGG

Pparγ, Peroxisome proliferator-activated receptor-gamma; *Agt*, Angiotensinogen; *Ace*, Angiotensin-converting enzyme; *Agtr1*, Angiotensin II receptor type 1; *Agtr2*, Angiotensin II receptor type 2; *Star*, Steroidogenic acute regulatory protein; *Hsd3b1*, 3β-Hydroxysteroid dehydrogenase type 1; *Cyp11a1,* Cholesterol side-chain cleavage enzyme; *Cyp21*, 21-Hydroxylase; *MR*, Mineralocorticoid receptor.

Table 3 Body weight, diet intake, and blood glucose in rat

	Initial Body Weight (g)	Final Body Weight (g)	Diet Intake (g/day)	Blood Glucose Level (mg/dL)
ND	202.95 ± 5.47^{ns}	509.03 ± 10.88^{c}	19.99 ± 0.39^{a}	89.83 ± 2.36^{a}
HD	201.82 ± 3.51^{ns}	633.27 ± 17.20^{a}	17.73 ± 0.46^{b}	93.8 ± 4.19^{a}
HDS	195.43 ± 8.61^{ns}	558.65 ± 9.58^{b}	17.02 ± 0.46^{b}	90.33 ± 1.89^{a}
HDJ	200.9 ± 4.73^{ns}	548.48 ± 6.41^{bc}	17.37 ± 0.42^{b}	80.13 ± 2.86^{b}

Values are given as mean ± SEM. Values with different superscripts in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test (a > b; ns, not significant). Six rats were assigned to

each group. ND; normal diet group, HD; high-fat diet group, HDS; high-fat diet with salt group, HDJ; high-fat diet with *doenjang* group.

Table 4 Na^+ and K^+ levels of urine, feces, and serum in rats.

	Urine (ppm)			Feces (ppm)			Serum (mEq/L)	
	Na^+	K^+	**Na^+/K^+**	Na^+	K^+	**Na^+/K^+**	Na^+	K^+
ND	1845.86± 134.35^{d}	4929.48± 461.13^{b}	0.42± 0.02^{c}	504.08± 41.96^{ns}	1098.18± 100.55^{a}	0.48± 0.37^{c}	134.7±0.85^{b}	4.34± 0.11^{b}
HD	2431.53± 158.41^{c}	12571.03± 469.02^{a}	0.19± 0.01^{d}	548.99± 98.73^{ns}	728.30± 68.08^{b}	0.43± 0.42^{c}	138.3±1.31^{a}	5.43± 0.17^{a}
HDS	9315.33± 32.78^{a}	1870.31± 67.18^{d}	4.46± 0.00^{a}	568.70± 2.70^{ns}	462.66 ± 39.11^{c}	1.59± 0.60^{a}	137.5± 0.43^{ab}	5.62± 0.12^{a}
HDJ	9426.76± 36.69^{a}	2183.75± 64.67^{c}	3.83± 0.00^{b}	614.53± 2.47^{ns}	828.92± 36.06^{b}	0.84± 0.77^{b}	136.8±1.05^{ab}	4.72± 0.21^{b}

Values are given as mean ± SEM. Values with different superscripts in the same column are significantly different ($p < 0.05$) by Duncan's multiple range test (a > b; ns, not significant). Six rats were assigned to each group. ND; normal diet group, HD; high-fat diet group, HDS; high-fat diet with salt group, HDJ; high-fat diet with *doenjang* group.

Figures

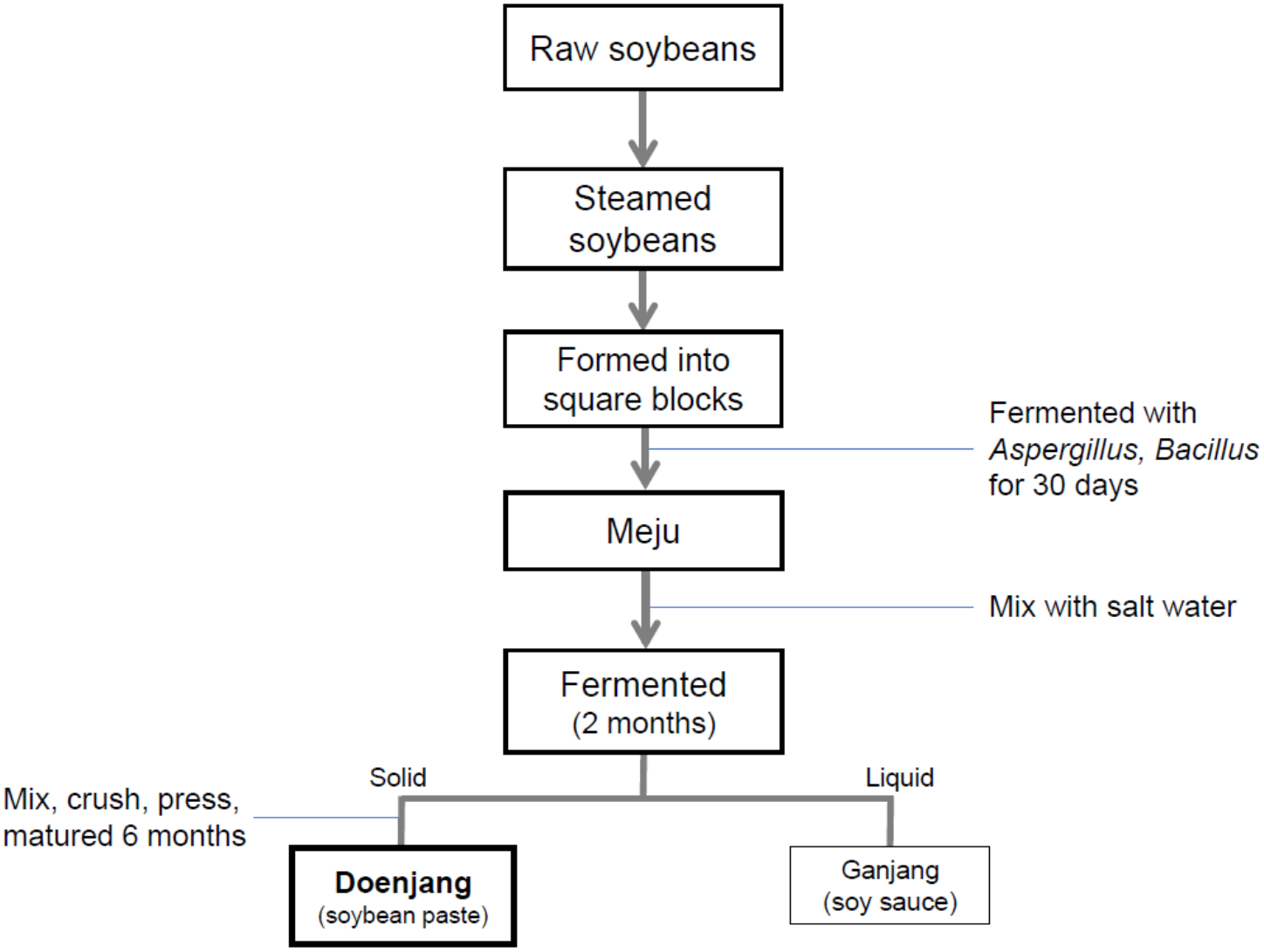

Figure 1

Manufacturing process diagram for Doenjang.

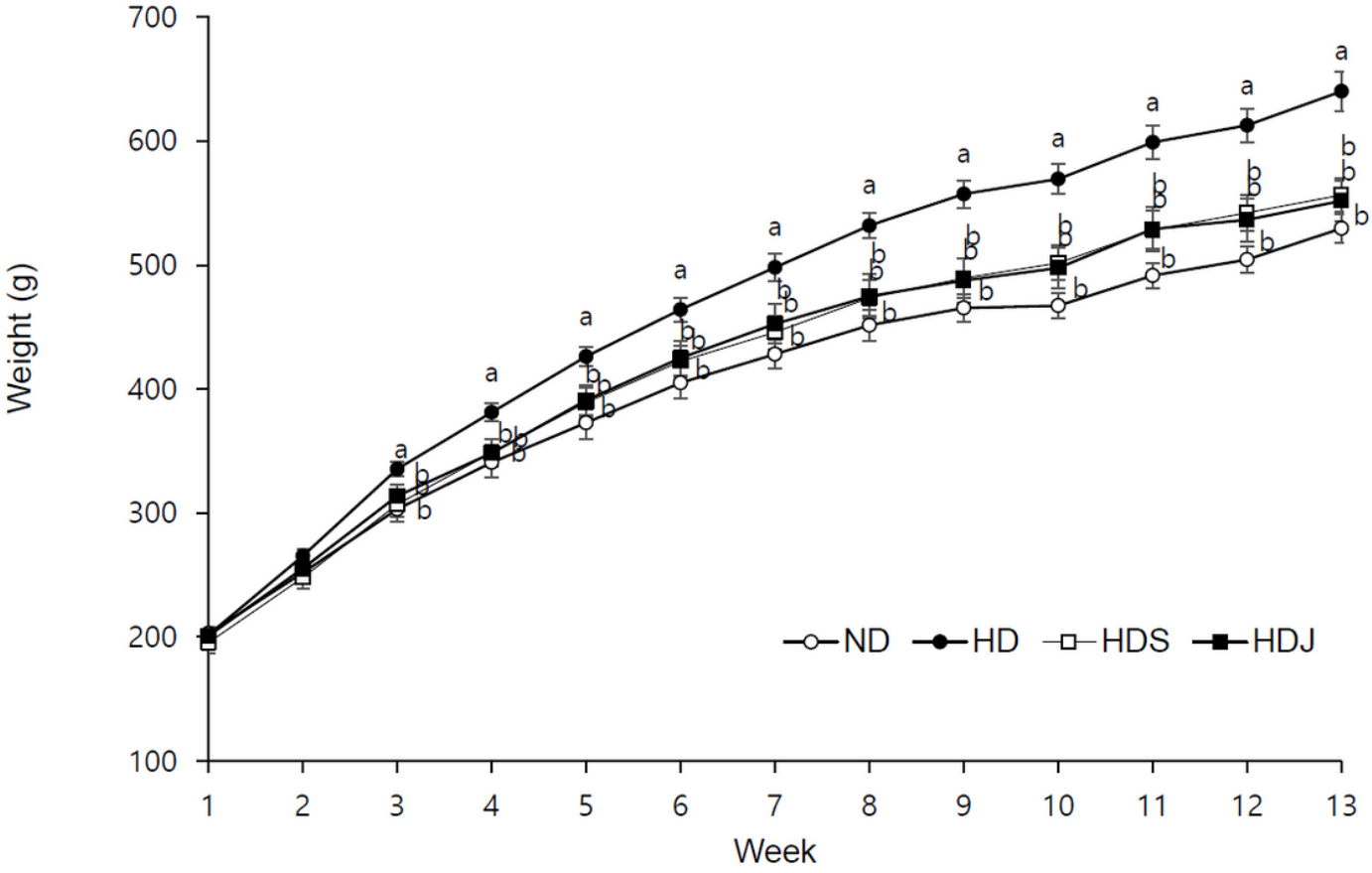

Figure 2

Growth curve of body weight. Body weight growth curve of the four groups of rats. The body weights of each group of rats recorded weekly are shown. Values are the mean ± SEM, with different letters significantly different ($p < 0.05$) by Duncan's multiple range test ($a > b$). Six rats were assigned to each group. ND; normal diet group, HD; high-fat diet group, HDS; high-fat diet with salt group, HDJ; high-fat diet with doenjang group.

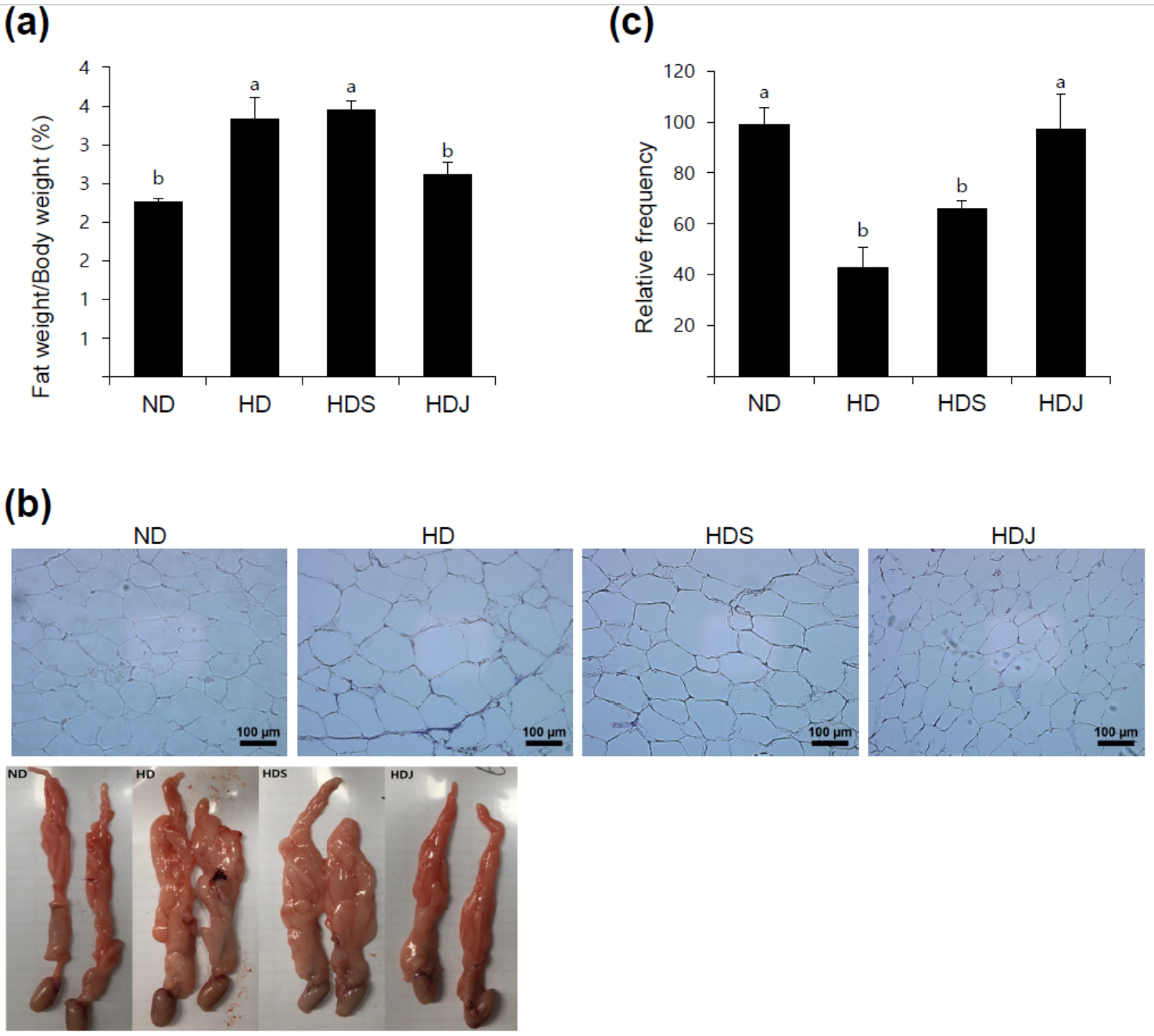

Figure 3

Epididymal fat weight and size of adipocyte in rats. (a) Epididymal fat-to-BW ratio (%), (b) pictures of representative gross epididymal fat and histology of fat section, (c) the ratio of the number of adipocytes to area. Values are given as mean ± SEM. Values with different superscripts are significantly different ($p < 0.05$) by ANOVA with Duncan's multiple range test (a > b). Six rats were assigned to each group. ND; normal diet group, HD; high-fat diet group, HDS; high-fat diet with salt group, HDJ; high-fat diet with doenjang group.

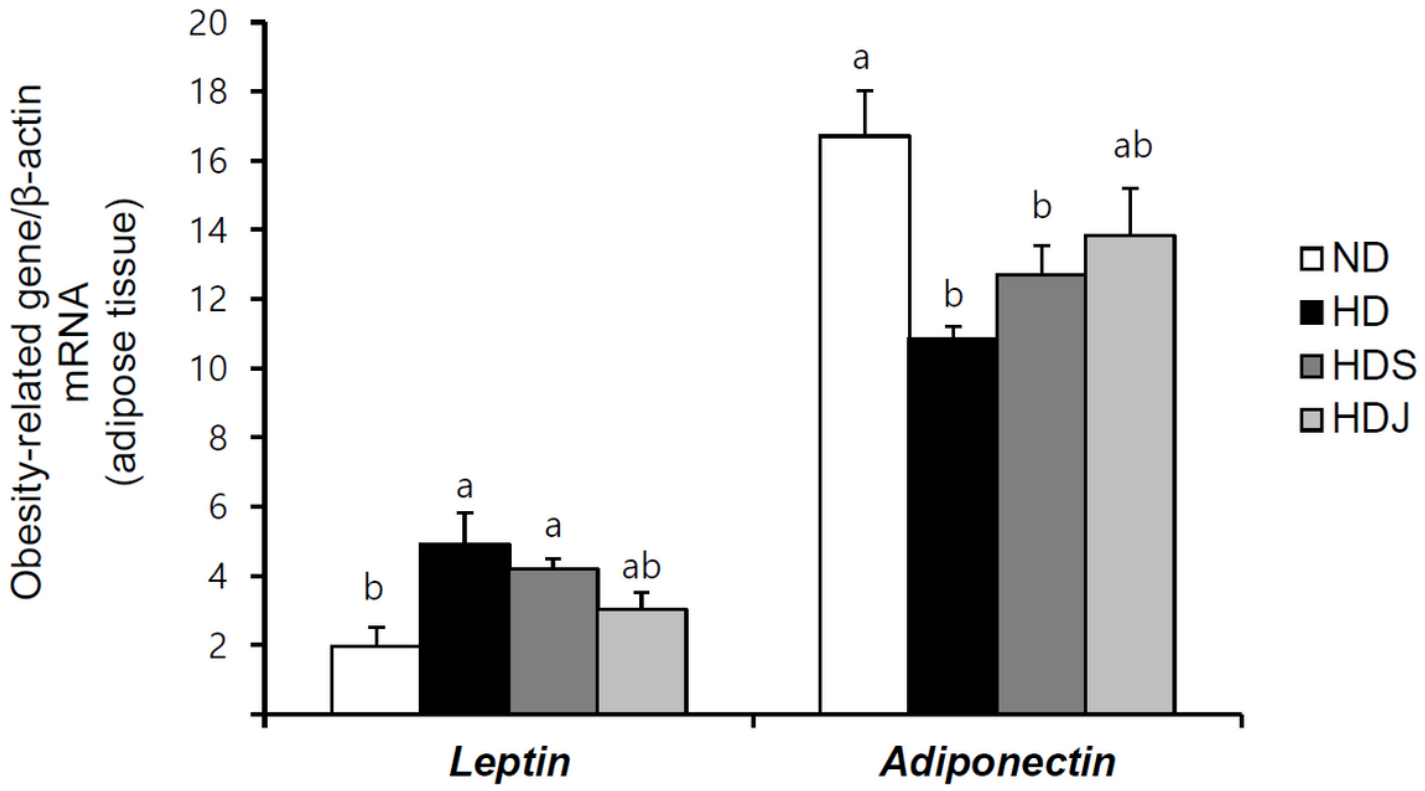

Figure 4

Expression of Obesity-related genes in epididymal fat. Values are given as mean ± SEM. Values with different superscripts are significantly different ($p < 0.05$) by ANOVA with Duncan's multiple range test (a > b). Six rats were assigned to each group. ND; normal diet group, HD; high-fat diet group, HDS; high-fat diet with salt group, HDJ; high-fat diet with doenjang group.

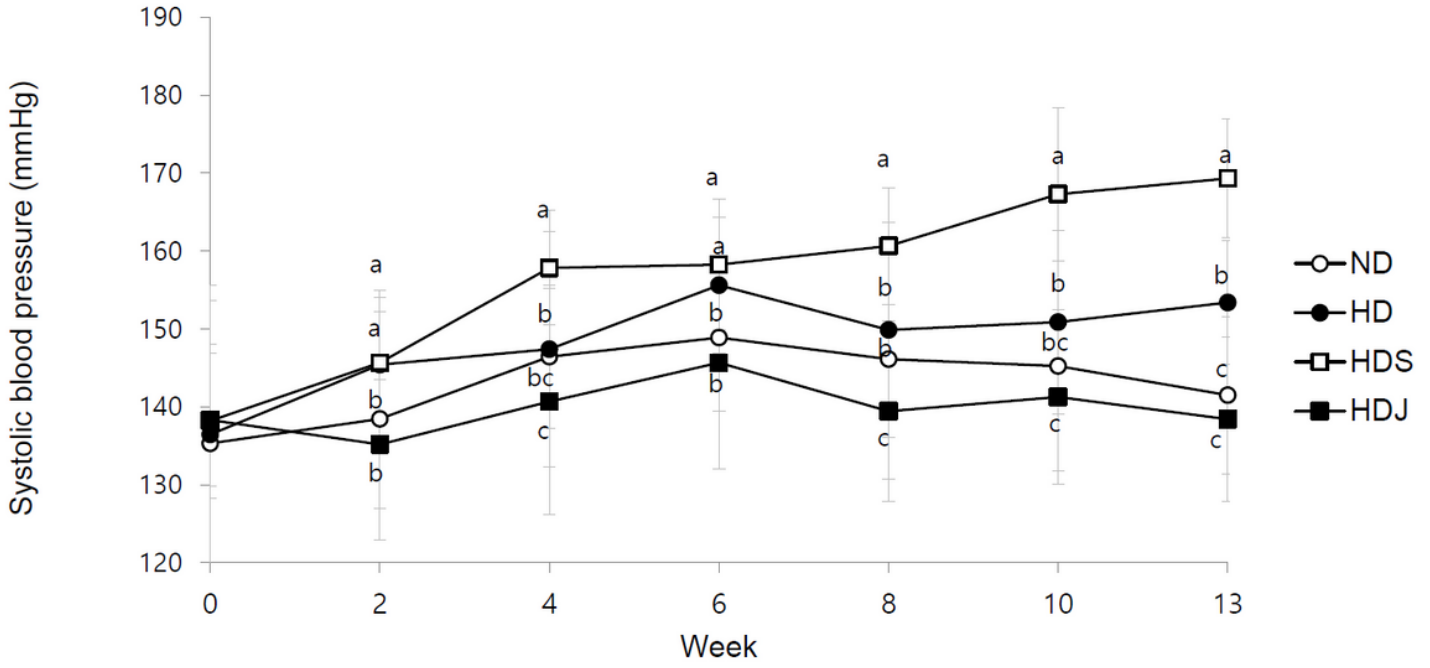

Figure 5

Systolic blood pressure for experimental period. Systolic blood pressure curve of the four groups of rats. The blood pressure of each group of rats recorded alternate weeks are shown. Values are the mean ± SD, with different letters significantly different ($p < 0.05$) by Duncan's multiple range test (a > b). Six rats were assigned to each group. ND; normal diet group, HD; high-fat diet group, HDS; high-fat diet with salt group, HDJ; high-fat diet with doenjang group.

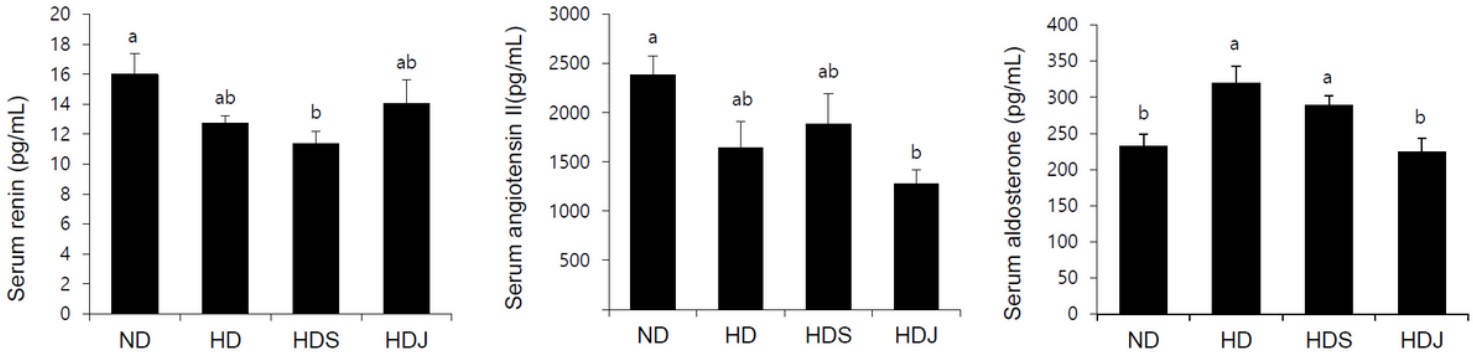

Figure 6

Renin, angiotensin II, and aldosterone levels in serum. Values are given as mean ± SEM. Values with different superscripts are significantly different ($p < 0.05$) by ANOVA with Duncan's multiple range test (a > b). Six rats were assigned to each group. ND; normal diet group, HD; high-fat diet group, HDS; high-fat diet with salt group, HDJ; high-fat diet with doenjang group.

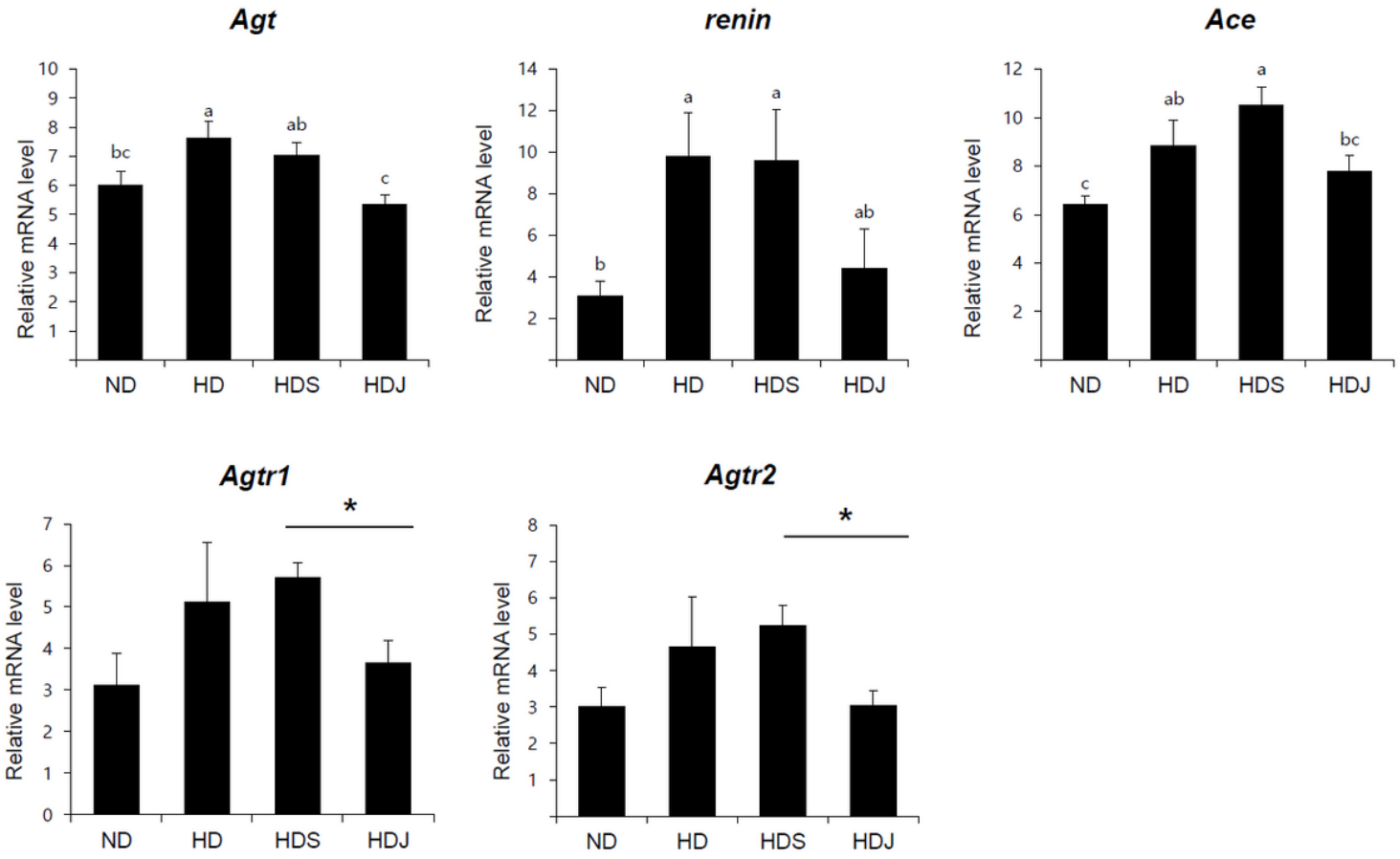

Figure 7

Effect of doenjang treatment on mRNA levels of RAS-related genes in white adipose tissue of rats. Values are given as mean ± SEM. Values with different superscripts are significantly different ($p < 0.05$) by ANOVA with Duncan's multiple range test (a > b). *$p < 0.05$ for HDS versus HDJ. Six rats were assigned to each group. Six rats were assigned to each group. ND; normal diet group, HD; high-fat diet group, HDS; high-fat diet with salt group, HDJ; high-fat diet with doenjang group. Agt; Angiotensinogen, Ace; Angiotensin-converting enzyme, Agtr1; Angiotensin II receptor type 1, Agtr2; Angiotensin II receptor type 2.

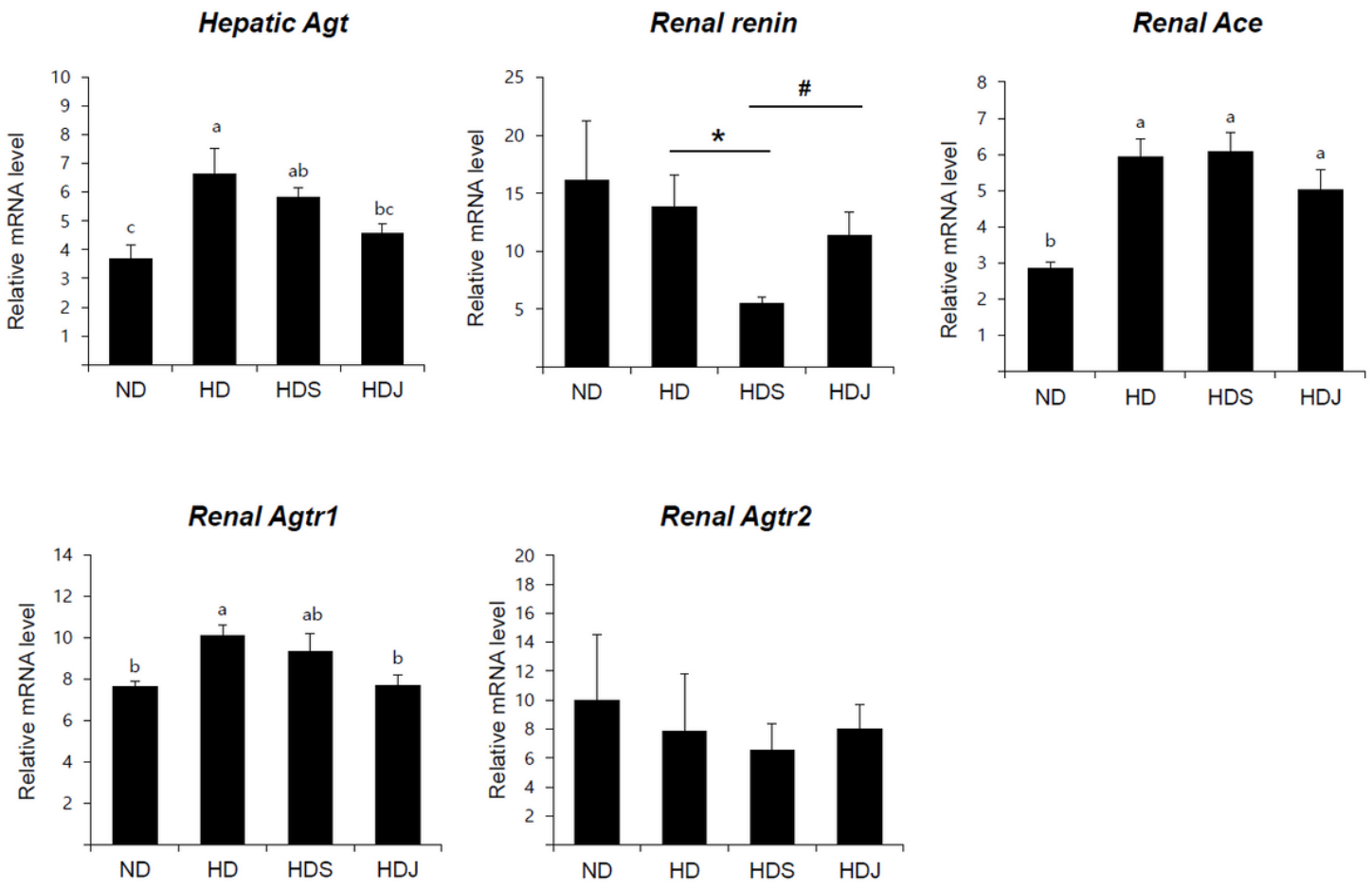

Figure 8

Effect of doenjang treatment on mRNA levels of RAS-related genes in liver and kidney of rats. Values are given as mean ± SEM. Values with different superscripts are significantly different ($p < 0.05$) by ANOVA with Duncan's multiple range test (a > b). *$p < 0.05$ for HD versus HDS, #$p < 0.05$ for HDS versus HDJ. Six rats were assigned to each group. Six rats were assigned to each group. ND; normal diet group, HD; high-fat diet group, HDS; high-fat diet with salt group, HDJ; high-fat diet with doenjang group. Agt; Angiotensinogen, Ace; Angiotensin-converting enzyme, Agtr1; Angiotensin II receptor type 1, Agtr2; Angiotensin II receptor type 2.

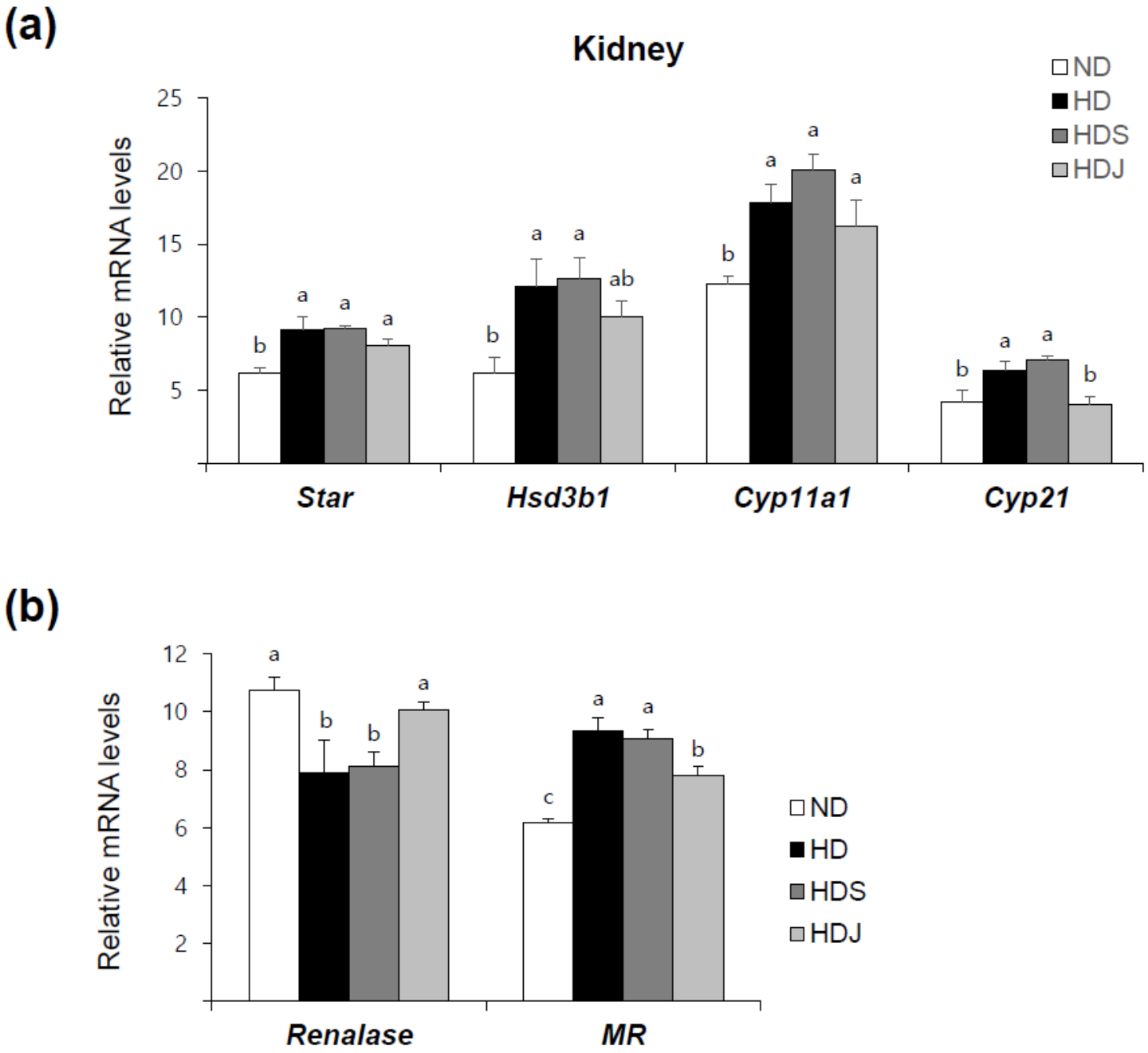

Figure 9

mRNA levels of Aldosterone related genes in kidney. (a) Aldosterone releasing factors, (b) MR and Renalase mRNA levels. Values are given as mean ± SEM. Values with different superscripts are significantly different ($p < 0.05$) by ANOVA with Duncan's multiple range test (a > b). Six rats were assigned to each group. ND; normal diet group, HD; high-fat diet group, HDS; high-fat diet with salt group, HDJ; high-fat diet with doenjang group. Star; Steroidogenic Acute Regulatory Protein, Hsd3b1; 3β-hydroxysteroid dehydrogenase type 1, Cyp11a1; cholesterol side-chain cleavage enzyme, Cyp21; 21-hydroxylase, MR; Mineralocorticoid receptor.

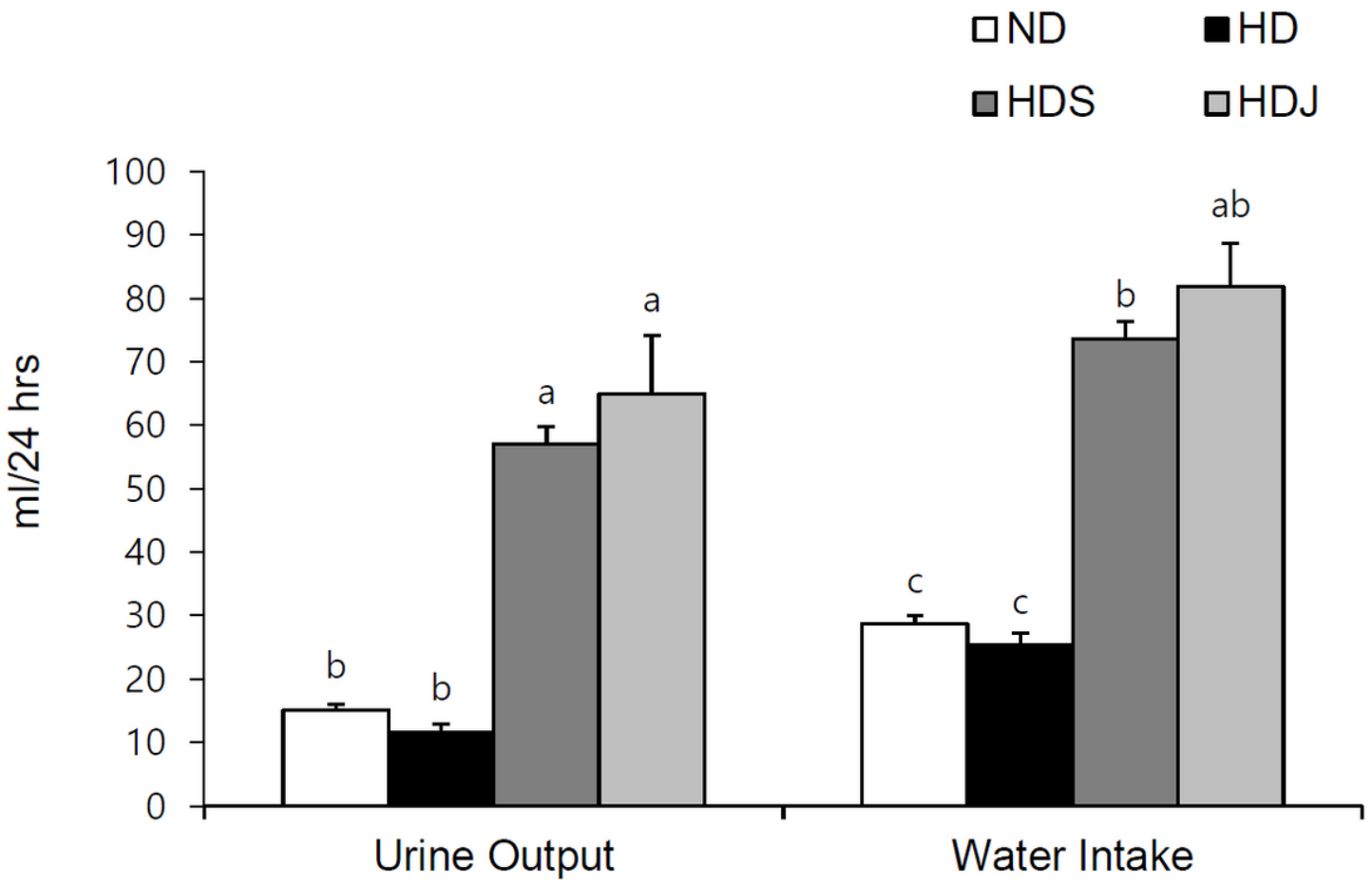

Figure 10

Urine output and water intake in 24 h metabolic cages. Values are given as mean ± SEM. Values with different superscripts are significantly different ($p < 0.05$) by ANOVA with Duncan's multiple range test (a > b). Six rats were assigned to each group. ND; normal diet group, HD; high-fat diet group, HDS; high-fat diet with salt group, HDJ; high-fat diet with doenjang group.

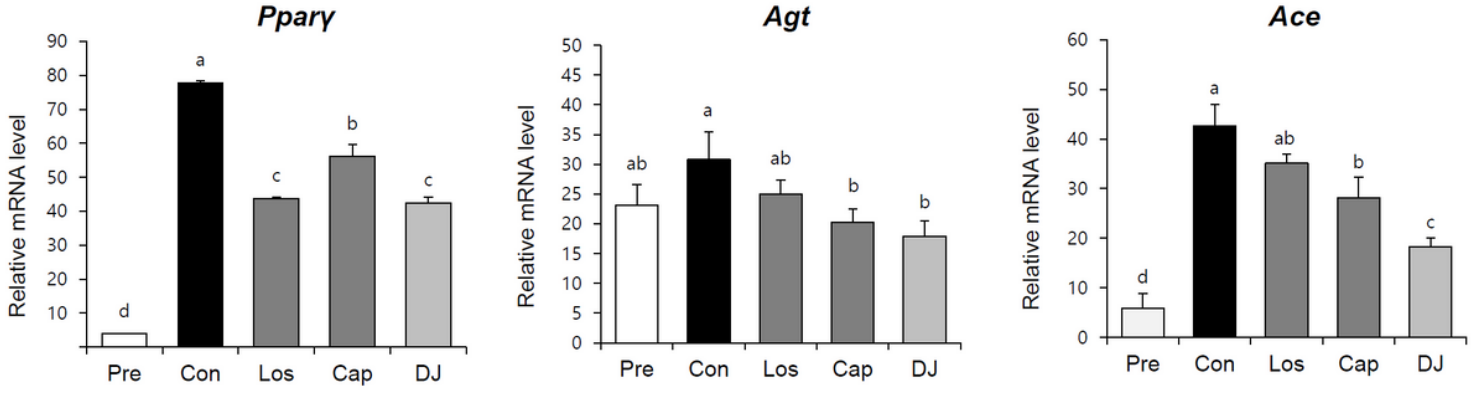

Figure 11

Effects of Doenjang extract on 3T3-L1 cell line. Values with different superscripts are significantly different ($p < 0.05$) by ANOVA with Duncan's multiple range test (a > b). Pre; preadipocytes, Con; adipocytes without

any treatment, Los; adipocytes treated with losartan (10-4 M), Cap; adipocytes treated with captopril (10-4 M), DJ; adipocytes treated with doenjang (0.4% salinity). Pparg; peroxisome proliferator-activated receptor gamma, Agt, Angiotensinogen, Ace; Angiotensin-converting enzyme.