Direct renin inhibitor, aliskiren, attenuates the progression of nonalcoholic steatohepatitis in the rat model

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Running head: Direct renin inhibitor attenuates the progression of NASH

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Abstract
Renin is a rate-limiting enzyme of renin angiotensin system (RAS), and several reports have shown that renin plays an important role in several pathological processes. Although RAS is known to play a pivotal role in the progression of nonalcoholic steatohepatitis (NASH), the role of renin is still obscure. The aim of the current study was to examine the effect of the clinically used direct renin inhibitor (DRI), aliskiren, on the progression of NASH in the rat model. The effects of DRI on the choline-deficient L-amino acid-defined (CDAA) diet-induced rat NASH model was examined in conjunction with the activated hepatic stellate cells (Ac-HSCs) and neovascularization, both are known to play important roles in the liver fibrosis development and hepatocarcinogenesis, respectively. DRI exerted a marked inhibitory effect against liver fibrosis development and glutathione-S-transferase placental form (GST-P)-positive preneoplastic lesions along with suppression of the Ac-HSCs and neovascularization in a dose-dependent manner. DRI also inhibited the hepatic expressions of transforming growth factor-beta 1 (TGF-beta 1), angiotensin-II (AT-II), and vascular endothelial growth factor (VEGF). These results indicated that renin played a pivotal role in the liver fibrosis development and hepatocarcinogenesis of NASH. Since DRI is already widely used in the clinical practice with safety, this drug may represent a potential new strategy against the progression of NASH in the future.
Key words: angiotensin-II, renin, nonalcoholic steatohepatitis, liver fibrosis, hepatocarcinogenesis.
**Introduction**

Nonalcoholic fatty liver disease (NAFLD) is increasingly recognized as a clinically important chronic liver disease, since nonalcoholic steatohepatitis (NASH) progresses to liver cirrhosis and finally hepatocellular carcinoma (HCC). The patients with NAFLD frequently have many clinical complications, such as obesity, type 2 diabetes mellitus, and insulin resistance. Although weight loss based on diet and exercise therapies is very effective and improves steatosis and inflammation in liver, many NAFLD patients cannot change their lifestyle and it is then necessary to employ additional therapy. Accordingly, efforts are currently directed worldwide at overcoming NAFLD, especially NASH. However, no widely accepted therapeutic modalities have been established in the clinical practice yet.

The pathogenesis of NASH is reportedly a multifactorial process. Angiogenesis; i.e., formation of new vessels by sprouting from the preexisting vasculature, is of central importance for embryonic development and organogenesis. In adults, physiological angiogenesis occurs during the female reproductive cycle and in wound healing. Pathological angiogenesis is observed in rheumatoid arthritis, psoriasis, diabetic retinopathy, fibrogenesis, and even in carcinogenesis. We previously reported that angiogenesis plays a pivotal role in the progression of NASH experimentally and clinically. We also revealed that antiangiogenic agents, such as monoclonal antibody against vascular endothelial growth factor (VEGF)-signaling, exerted potent...
inhibitory effects against the liver fibrosis and hepatocarcinogenesis\textsuperscript{14, 15}. Several antiangiogenic agents have already been employed in the clinical practice\textsuperscript{16}. A multikinase inhibitor, including VEGF receptors, sorafenib, has prolonged the overall survival of patients with advanced HCC, and this drug is already approved in many countries for treatment of HCC\textsuperscript{17, 18}. It is very likely that sorafenib will become the standard therapeutic agent of cure for the advanced stages of HCC in the near future, but long-term administration of this agent to cirrhotic patients may lead to severe side effects since the drug metabolism is usually hypoactive in those patients. As such, almost all patients show adverse reactions with sorafenib such as hand-foot syndrome, and some symptoms are very severe\textsuperscript{19, 20}. Furthermore, these recently developing agents are very expensive\textsuperscript{21}. An alternative approach may be to find a clinically available compound that also exhibits antiangiogenic activity, of which the safety of long-term administration has been proven.

The renin-angiotensin system (RAS) is a key mediator in regulation of the arterial blood pressure and body fluid homeostasis, and it plays an important role in the regulation of local hemodynamics in several organs\textsuperscript{22, 23}. We and other groups have shown that angiotensin-II (AT-II), an octapeptide produced via enzymatic cleavage of angiotensin-I by angiotensin-I converting enzyme (ACE), plays an important role in the progression of chronic liver diseases, including NASH\textsuperscript{24-26}. Several studies have demonstrated that AT-II exerted a marked proangiogenic activity. We and another group have shown that suppression of AT-II by a
clinically used AT-II type-I (AT1) receptor blocker (ARB) significantly attenuated liver fibrosis development and hepatocarcinogenesis in the rat NASH model along with attenuation of neovascularization in the liver\textsuperscript{24, 25, 27}. AT-II is also known as a key player in the activated hepatic stellate cells (Ac-HSCs), which play an important role in liver fibrosis development\textsuperscript{28}. Suppressive effects of ACE-I and ARB against liver fibrogenesis were noticed along with attenuation of the Ac-HSCs\textsuperscript{29, 30}. These results indicate that the suppressive effects of ACE-I and ARB were achieved by coordination of antiangiogenesis and Ac-HSCs inhibition. In addition to AT-II, recent studies have revealed that renin also plays an important role in the fibrosis development in the kidney and heart\textsuperscript{31, 32}. The direct renin inhibitor (DRI), aliskiren, has recently become available in the clinical practice as a new type of antihypertensive agents. It seems to inhibit production of AT-II and other angiotensin derivatives by blocking the first rate-limiting enzyme of RAS\textsuperscript{33, 34}. Similar to ACE-I and ARB, several investigators have demonstrated that DRI exerted a marked suppressive effect against the fibrosis development in the kidney and heart\textsuperscript{32, 33, 35}. However, the role of renin and the effect of DRI on the progression of chronic liver diseases have not been elucidated yet.

In the current study, we examined the effects of DRI on liver fibrosis and hepatocarcinogenesis in the rat NASH model induced by feeding rats on a choline-deficient, L-amino acid-defined diet (CDAA)\textsuperscript{27}. We also attempted to investigate the possible mechanisms involved.
Materials and Methods

Animals and reagents

A total of 40 male 6-week-old Fischer 344 rats were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). They were housed under controlled temperature conditions and relative humidity, with 10-15 air changes/h and light illumination for 12 h/day. Aliskiren, as DRI, was purchased from Novartis Inc. (Tokyo, Japan). Recombinant rat and human renin were purchased from Ana Spec, Inc. (Fremont, CA, USA). Conventional chemical agents were purchased from Nacalai Tesque (Kyoto, Japan). The human umbilical vascular endothelial cells (HUVECs) were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and Kurabo (Osaka, Japan). The cells were grown in a supplier-recommended medium.

Animal treatment

The experimental period in all experiments was 18 weeks. Group(G) 1 consisted of untreated rats that served as the CDAA-fed control group. The rats in G2 and G3 received a CDAA diet in addition to 50 mg/kg/day and 100 mg/kg/day of DRI for 12 weeks starting from week 6 to the end of experiment by gavage, respectively. In G4, the rats received a choline-sufficient L-amino acid-defined (CSAA) diet as a negative control. The animals were allowed free access to food and water throughout the acclimation and experimental protocols. Several serum
markers, such as alanine aminotransferase (ALT), were assessed by routine laboratory methods. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals.

Histological and immunohistochemical staining and quantification

In all the experimental groups, the first liver section was stained with hematoxylin and eosin for routine histological examination. Another section was stained with Sirius red for detection of fibrosis development. Immunohistochemical staining for the α-smooth muscle actin (α-SMA) (Dako, Kyoto, Japan) and the enzyme-altered preneoplastic lesions; namely, the placental form of glutathione-S-transferase (GST-P) (MBL Co. Ltd., Nagoya, Japan), was performed as described previously. Semiquantitative analyses of fibrosis development and immunopositive cell area of α-SMA and GST-P were carried out with Adobe Photoshop software and National Institutes of Health image software in 6 ocular fields (magnification ×40) per specimen of 10 rats as described elsewhere with minor modification 36.

Extracellular signal-regulated kinase 1/2 phosphorylation and the mRNA expressions of several indices in the liver

The hepatic extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation was determined using 200 mg of frozen liver samples. After equalization of the protein contents, the phosphorylated-ERK1/2
(p-ERK) and the total-ERK1/2 (t-ERK) were determined by ERK ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions.

The transforming growth factor-β (TGF-β), procollagen I, VEGF, platelet/EC adhesion molecule (PECAM/CD31), which is widely used as a marker of neovascularization, AT-II and (pro) renin receptor (PRR) mRNA expression levels in the liver were measured by real time PCR as described previously. RNA was extracted from the powdered frozen liver tissues using an RNeasy mini kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer’s instructions. The expression level of mRNA was quantified with real-time PCR by SYBR Green on a Step One Plus sequence detection system (Applied Biosystems Inc.). The specific primers for the target genes were as follows: TGF-β forward, 5-CGG CAG CTG TAC ATT GAC TT-3 and reverse, 5-AGC GCA CGA TCA TGT TGG AC-3; α1(I)-procollagen forward, 5-AAA GCA GAA ACA TCG GAT TTG G-3 and reverse, 5-CGT GTC ATC CCT TGT GCC GCA-3; VEGF forward, 5-GAG GAA AGG GAA AGG GTC AAA A-3 and reverse, 5-CAC AGT GAA CGC TCC AGG ATT-3; CD31 forward, 5-GGC GTC CTG TCC GGA ATC-3 and reverse, 5-AGA ACT CCT GCA CAG TGA CGT ATT-3; AT-II forward, 5-CAA GTC CTG AAG ATG GCA GCT-3 and reverse, 5-AAG GTC AGA ACA TGC AAG GGA A-3, PRR forward, 5-GAGGCAGTGACCCTCAACAT-3 and reverse 5-CCCTCCTCACAACAACAGGT-3. GAPDH was used as an endogenous
control, and the results of the target genes were determined using the comparative threshold (Ct) value.

Statistical analysis

To assess the statistical significance of inter-group differences in the quantitative data, Bonferroni’s multiple comparison test was performed after one-way ANOVA. This was followed by Barlett’s test to determine the homology of variance.

Results

General findings in the CSAA- and CDAA-treated rats

The data pertaining to the effective numbers of rats, final body and relative liver weights in all experimental groups are shown in Table 1. The DRI-treated groups at a dose of the 50 mg/kg/day and 100 mg/kg/day (G2 and G3, respectively) did not show any alteration in several indices, such as the ALT level and albumin (Alb). Neither ascites nor other organ abnormalities were observed at the end of the experiment in any of the groups. In the CDAA model, the final body and relative liver weights of the CDAA-treated rats (G1, G2, and G3) were less than those of the CSAA-treated rats (G4), as stated in previous reports. The relative liver weights in G2, G3 and G4 were greater than those in G1.

Effects of DRI on the liver fibrosis development
As shown in Fig. 1A, the rats of G2 and G3 showed a significant inhibitory effect against liver fibrosis development as compared to the control group (G1) in a dose-dependent manner. Semiquantitative analysis revealed that the fibrosis area in G3 was almost one-third that in G1 (Fig. 1B). We then carried out an immunohistochemical analysis of α-SMA to examine the effect of DRI on HSCs activation during liver fibrosis development (Fig. 1C). The Ac-HSCs, which express α-SMA, were drastically reduced in the liver of the DRI-treated groups (G2 and G3). This was similar to the effect on liver fibrosis development (Fig. 1D).

As compared to the rats that received a CDAA diet, the DRI-treated groups showed a decrease in TGF-β1 and α1(1)-procollagen mRNA expressions in the liver, and the inhibitory effects of DRI on the mRNA expressions were almost parallel to the inhibition of liver fibrosis (Fig. 2A and 2B, respectively). We also examined the effect of DRI on the liver local RAS inhibition. As shown in Fig. 2C, similar to the effects on the liver fibrosis development, the DRI-treated groups (G2 and G3) showed a significant inhibitory effect on hepatic AT-II expression as compared to the control group (G1) in a dose-dependent manner. On the other hand, the treatment with DRI did not affect the PRR mRNA expression in the liver (Fig. 2D).

*Effects of DRI on the development of preneoplastic lesions, neovascularization, and ERK 1/2 phosphorylation in the liver*
We examined the effect of DRI on the GST-P-preneoplastic lesions in conjunction with neovascularization. As shown in Fig. 3A and 3B, similar to the effect on liver fibrosis inhibition, DRI-treatment (G2 and G3) exerted a significant inhibitory effect on the GST-P-positive preneoplastic lesion development as compared to the control group (G1) in a dose-dependent manner. In the CSAA-treated rats, no GST-P-positive lesions were found. To determine whether the inhibitory effect of DRI against the preneoplastic lesion development was accompanied by suppression of neovascularization, we examined the mRNA expression levels of CD31 and VEGF. The treatment with DRI significantly attenuated the CD31 and VEGF mRNA expressions in the liver along with inhibition of GST-P-induced preneoplastic lesions (Figs. 4A and 4B, respectively). We also examined the ERK 1/2 phosphorylation in the liver. The treatment with DRI significantly attenuated the ERK 1/2 phosphorylation in the liver along with inhibition of neovascularization (Fig. 4C).

Discussion

In the current study, we first showed that, in addition to AT-II, renin played an important role in the progression of NASH, and that DRI markedly attenuated both liver fibrosis development and hepatocarcinogenesis. It is well known that the Ac-HSCs play a pivotal role in the liver fibrosis development. Renin augmented the proliferation,
phosphorylation of ERK 1/2, TGF-β1 mRNA, and α1(1)-procollagen mRNA in the Ac-HSCs. The hepatic levels of TGF-β1 and α1(1)-procollagen, which are mainly produced in the Ac-HSCs, were suppressed by treatment with DRI. These results indicated that renin directly acted on the Ac-HSCs, and that the anti-fibrotic effect of DRI was, at least partly, achieved via suppression of HSCs activation. Interestingly, DRI did not suppress these parameters in the Ac-HSCs in vivo. Furthermore, renin significantly increased the ratio of p-ERK/t-ERK. Similar to the proliferation assay results, DRI did not show any marked inhibitory effect on the Ac-HSCs. We also observed that the TGF-β1 and α1(1)-procollagen mRNA expressions in the Ac-HSCs were significantly increased by treatment with renin, whereas DRI did not exert any significant inhibitory effects (data not shown). The exact reason of discrepancy is not clear at this time, but it may be due to the pharmaceutical action of DRI. DRI inhibits AT-I generation from angiotensinogen by direct renin inhibition, and it also decreases AT-II production, but not through direct interaction between renin and PRR\textsuperscript{31, 37-39}. In accordance with this mechanism of action, our study revealed that DRI significantly suppressed the AT-II mRNA in the liver, whereas no decrease could be observed in the PRR level. Since DRI did not block the interaction between renin and PRR, the discrepancies between the in-vivo and in-vitro results could be observed in the current study. Further studies are required using direct inhibitor of PRR in the future.
In addition to the liver fibrosis development, DRI markedly attenuated the preneoplastic lesion development. We focused on the role of neovascularization in the progression of NASH. Angiogenesis is now recognized to play an important role in many physiological and pathological events. Recent studies have revealed that angiogenesis has already begun at a very early stage when the neoplastic cells contained just a couple of hundreds cells. We previously reported that angiogenesis significantly increased during hepatocarcinogenesis, even in the CDAA-fed rat NASH model, and neovascularization was evident mainly in the GST-P-positive lesions as compared with the adjacent areas. Among the identified angiogenic factors, VEGF is the most potent factor in angiogenesis. Furthermore, the VEGF expression stepwise increased during hepatocarcinogenesis, and suppression of the VEGF signaling cascade attenuated these pathological sequences. We previously reported that VEGF-mediated neovascularization played a pivotal role in the progression of NASH in the animal model as well as in the clinical practice. It has been reported that AT-II induced neovascularization, and we observed that renin also significantly increased the ECs proliferation in the current study. In the ECs, similar to the findings in the Ac-HSCs, renin at a dose of $10^{-6}$ M stimulated the ECs proliferation, but the renin-induced ECs proliferation was not suppressed by very high dose of DRI at $10^{-4}$ M (data not shown).
Moreover, DRI suppressed the VEGF-mediated neovascularization in the liver. The antiangiogenic effects of DRI should contribute to the suppressive effects against hepatocarcinogenesis in NASH.

Although previous studies conducted to determine the molecular processes associated with fibrosis and angiogenesis were performed independently, recent studies have revealed that both biological phenomena emerged synergistically\textsuperscript{14, 43}. We and other groups reported that neovascularization significantly increased during the liver fibrosis development \textsuperscript{14, 44, 45}. As in the ECs, we have shown that renin could act on the Ac-HSCs in the current study. Accordingly, it is possible that augmentation of renin-mediated neovascularization may also play some role in the development of liver fibrosis development.

In this study, we used the CDAA model to elucidate the effects of DRI on the progression of NASH. This CDAA model has several critical disadvantages as a NASH model, although the histological progression features are very similar to those in the human NASH\textsuperscript{46}. This CDAA diet model does not induce typical features of the human NASH such as obesity, glucose intolerance, and insulin resistance. To examine the precise pharmacological action of any drug, it is important to examine its therapeutic effect under the condition of insulin resistance. We previously investigated the effect of ARB on the CDAA-induced liver fibrosis development in the Otsuka Long-Evans Tokushima fatty (OLETF) rats, which commonly have obesity, diabetes mellitus, and insulin resistance\textsuperscript{27}. The therapeutic effect of DRI in the CDAA-induced
OLETF rats should be elucidated in the future. Although the inflammation in the CDAA-diet model was not as severe as that in other necro-inflammation predominant models such as CCl4, there was some inflammatory reaction in this model especially in the early stage as described previously. Consequently, the ALT level in the CDAA-treated rats was significantly higher than that in the control (Table 1). Many published studies were conducted using the CDAA-diet model as a NASH model. Accordingly, we assume that we can use the term NASH for this model as it shows histological sequences similar to those noticed in chronic liver diseases.

Our knowledge of the multistage nature of hepatocarcinogenesis with initiation, promotion, and progression has been derived from studies using animal models. Considering the future possible clinical application, a better model may be created by administering the tested-agent from the promotion stage rather than from the beginning of the experiment. We therefore administered DRI from week 6; i.e., the promotion stage in the current study. Nevertheless, it is important to perform a long-term experiment in the future to determine whether or not DRI can really prevent HCC development. Furthermore, it would be important to examine whether DRI attenuated the cell transformation or influenced the preneoplastic lesion growth. As well as hepatocarcinogenesis, it is also much better that the DRI administration started from week 6 rather than from the beginning of the study to elucidate the therapeutic effect against the liver fibrosis development.
Since most of the patients already have chronic liver damage to some extent in the clinical practice, our current protocol is more likely to elucidate such possibility for future clinical application.

Recently, it has been reported that DRI also suppressed the CCl4-induced liver fibrosis along with inhibition of oxidative stress markers and inflammation. These results indicated that the anti-fibrotic effect of DRI on liver fibrosis development was not specific for an animal-model but a general phenomenon, and the suppressive effect of DRI on the oxidative stress also played an important role. In the current study, the ALT level was not significantly altered by DRI, indicating that the inhibitory effect of DRI in this model was mainly due to inhibition of AT-II-mediated HSCs activation and neovascularization, rather than suppression of inflammatory reaction. However, another report has shown that DRI could suppress the inflammation through inhibition of several cytokines such as TNF-α in the CCl4-induced liver fibrosis. Similar controversial results have been reported about the liver fibrosis inhibition by ARB. The reason of this discrepancy was not clear at this time. The CCl4-induced liver fibrosis showed more severe inflammation than that in our CDAA-induced model. This may be one of the reasons explaining why the anti-inflammatory effect of DRI played a significant role in the CCl4-induced liver fibrosis animal model but not in our model. Moreover, DRI did not significantly suppress the steatosis in the liver. This indicated that the inhibitory effect of DRI in this model was mainly due to inhibition of AT-II-mediated HSCs activation and neovascularization.
rather than the suppression of fat metabolisms although the mechanistic insights are not clear at this time. Further new projects are required to elucidate the exact mechanistic insights.

In summary, we have shown herein that renin plays an important role in the progression of NASH. The treatment with DRI significantly inhibited the liver fibrogenesis and carcinogenesis, at least partly, via suppression of the Ac-HSC and neovascularization, respectively. It is noteworthy that the inhibitory effect of DRI against the progression of NASH could be achieved at clinically comparable low doses. Since DRI is already widely used in clinical practice with great safety, it may represent a potential new therapeutic strategy against the progression of NASH in the near future.

Disclosures
The authors have no conflict of interest and financial support for this manuscript.
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Figure legends:

Figure 1.
Effect of the direct rennin inhibitor (DRI) on the liver fibrosis development.
A, C: Representative features of microphotographs and immunohistochemical analysis of the α-smooth muscle action (α-SMA) expression in the liver, respectively. B and D: Semiquantitative analysis of the liver fibrosis development and α-SMA, respectively. No fibrosis development could be observed in the choline-sufficient L-amino acid (CSAA)-treated control rats (G4). The choline-deficient L-amino acid (CDAA)-treatment showed an extensive fibrosis development along with fatty accumulation, and the DRI-treated groups at a dose of 50 mg/kg/day and 100 mg/kg/day (G2 and G3, respectively) showed a significant inhibitory effect against liver fibrosis development as compared to the control group (G1) in a dose-dependent manner. The α-SMA-positive lesions were almost of similar magnitude to the fibrosis area. The data represent the mean ± SD (bars; n =10). * indicates statistically significant difference between the indicated experimental groups (p<0.01). G1: CDAA-treated control group. G2 and G3: CDAA with DRI-treated groups (50 mg/kg/day and 100 mg/kg/day, respectively). G4: CSAA-treated group.

Figure 2.
Effects of DRI on the transforming growth factor (TGF)-β1, α1(1)-procollagen, angiotensin II (AT-II), and pro-renin receptor (PRR) mRNA expressions in the liver. A, B: DRI showed a decrease in TGF-β1 and α1(1)-procollagen mRNA expressions in the liver, and the inhibitory effects of DRI on the mRNA expressions were almost parallel to the inhibition of liver fibrosis. C: DRI-treated groups (G2 and G3) showed significant inhibition of the hepatic AT-II expression as compared to the control group (G1) in a dose-dependent manner. D: The treatment of DRI did not affect the PRR mRNA expression in the liver. The data represent the mean ± SD (bars; n = 10). * indicates statistically significant difference between the indicated experimental groups (p< 0.01). G1: CDAA-treated control group. G2 and G3: CDAA with DRI-treated groups (50 mg/kg/day and 100 mg/kg/day, respectively). G4: CSAA-treated group.

Figure 3.

Effects of DRI on the preneoplastic lesions in the liver.

A: Representative photomicrographs of glutathione-S-transferase placental form (GST-P)-positive preneoplastic lesions in the liver. B: Semiquantitative analysis revealed that DRI-treatment (G2 and G3) exerted significant inhibitory effect against the GST-P-positive preneoplastic lesion development as compared to the CDAA-treated group (G1) in a dose-dependent manner. In the CSAA-treated rats (G4), no GST-P-positive lesions were found. *and ** indicate statistically
significant differences between the indicated experimental groups ($p<0.01$ and $p<0.05$, respectively). G1: CDAA-treated control group. G2 and G3: CDAA with DRI-treated groups (50 mg/kg/day and 100 mg/kg/day, respectively). G4: CSAA-treated group.

**Figure 4.**

Effect of DRI on the hepatic neovascularization and phosphorylation of ERK1/2 in the liver.

The expressions of both CD31 (A) and vascular endothelial growth factor (VEGF) (B) significantly increased in the CDAA-diet group (G1) as compared with the CSAA-fed control group (G4). Treatment with DRI (G2 and G3) markedly suppressed the expressions of both CD31 and VEGF in comparison with G1 in a dose-dependent manner. C: The treatment with DRI significantly attenuated ERK 1/2 phosphorylation in the liver along with inhibition of hepatic neovascularization. * and ** indicate statistically significant differences between the indicated experimental groups ($p<0.01$ and $p<0.05$, respectively). G1: CDAA-treated control group. G2 and G3: CDAA with DRI-treated groups (50 mg/kg/day and 100 mg/kg/day, respectively). G4: CSAA-treated group.
### Table 1. Characteristic features of the experimental groups

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The data represent the mean ± SD. †, ‡: Statistically significant as compared to G1 (†p<0.05, ‡p<0.01)
Supplemental Figure.