Research Article

Herbal Supplement Ameliorates Cardiac Hypertrophy in Rats with CCl4-Induced Liver Cirrhosis

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We used the carbon tetrachloride (CCl4) induced liver cirrhosis model to test the molecular mechanism of action involved in cirrhosis-associated cardiac hypertrophy and the effectiveness of Ocimum gratissimum extract (OGE) and silymarin against cardiac hypertrophy. We treated male wistar rats with CCl4 and either OGE (0.02 g/kg B.W. or 0.04 g/kg B.W.) or silymarin (0.2 g/kg B.W.). Cardiac eccentric hypertrophy was induced by CCl4 along with cirrhosis and increased expression of cardiac hypertrophy related genes NFAT, TAGA4, and NBP, and the interleukin-6 (IL-6) signaling pathway related genes MEK5, ERK5, JAK, and STAT3. OGE or silymarin co-treatment attenuated CCl4-induced cardiac abnormalities, and lowered expression of genes which were elevated by this hepatotoxin. Our results suggest that the IL-6 signaling pathway may be related to CCl4-induced cardiac hypertrophy. OGE and silymarin were able to lower liver fibrosis, which reduces the chance of cardiac hypertrophy perhaps by lowering the expressions of IL-6 signaling pathway related genes. We conclude that treatment of cirrhosis using herbal supplements is a viable option for protecting cardiac tissues against cirrhosis-related cardiac hypertrophy.

1. Introduction

Patients with advanced cirrhosis have consistently been diagnosed with cardiac dysfunction under the condition of hyperdynamic circulation [1]. Increased cardiac output and reduced systemic vascular resistance are both signs of this condition [2–4]. Although cardiac dysfunction in patients with cirrhosis and potential clinical implications have long been known [5], little is understood regarding the molecular mechanism of action involved in cirrhosis-associated alteration in cardiac structure and function, especially cardiac hypertrophy.

Cirrhosis is known as a possible cause of portal vein constriction which may induce the activation of vasopressin,
angiotensin II (Ang II), and the sympathetic nervous system
[6]. Cardiac hypertrophy is induced by such direct mecha-
nical wall stress as well as paracrine/autocrine factors such as
Ang II, which in turn activates specific signaling pathways,
for instance, mitogen-activated protein kinases (MAPKs)
and calcineurin. These can cause cardiac hypertrophy and
increase of related gene expressions, such as proto-oncogenes
c-Fos and c-JUN, genes which encode atrial natriuretic
peptide (ANP) and B-type natriuretic peptide (BNP), and
structural genes β-myosin heavy chain (β-MHC) and skeletal
α-actin [7]. Ang II is associated with increased plasma levels
of proinflammatory cytokines such as interleukin-6 (IL-6)
[8], which is an effective stimulator of the Janus kinase/signal
transducer and activator of transcription (JAK/STAT) path-
way in cardiac hypertrophy [7]. However, the role of
these protein markers and transcriptional factors in cardiac
hypertrophy and remodeling in vivo has not been examined
in cirrhosis-associated hypertrophy.

Carbon tetrachloride (CCL4) is frequently used to induce
experimental cirrhosis in rats [9]. This model has recently
been used to investigate the role of lipophilic bile acids and
examine cardiac gene expression profiles in cirrhotic
cardiomyopathy [10, 11]. Silymarin, a standardized extract
of the milk thistle (Silybum marianum L. Gaertner), contains
three biochemicals: silybin, silydianin, and silychristin and
has a long tradition as a herbal remedy [12]. Ocimum
gratissimum extract (OGE), a commonly used herb in folk
medicine, is rich in antioxidants and possesses many ther-
apeutic functions [13–21]. Both herbal extracts have been
shown using the CCL4 model to inhibit liver cirrhosis [22].
Therefore the motive for this experiment is to use the CCL4-
induced liver cirrhosis model to understand the molecular
mechanism of action involved in cirrhosis-associated cardiac
hypertrophy, as well as to test effectiveness of silymarin and
OGE against cardiac damage and hypertrophy.

2. Materials and Methods

2.1. Preparation of OGE. Leaves of Ocimum gratissimum
were harvested and washed with distilled water followed
by homogenization with distilled water using polytron.
The homogenate was incubated at 95°C for 1 hour (h)
and then filtered through two layers of gauze. The filtrate
was centrifuged at 20,000 g, 4°C for 15 minutes (min)
to remove insoluble pellets and the supernatant (OGE)
was thereafter collected, lyophilized, and stored at −20°C
until use. The final extract (OGE) was composed of 11.1%
polyphenol (including 0.03% catechins, 0.27% caffeic acid,
0.37% epicatechin, and 3.27% rutin).

2.2. Animals and Treatment. Forty male wistar rats weighing
200–240 g were purchased from the National Animal Center
and housed in conventional cages with free access to water
and rodent chow at 20–22°C with a 12-hour light-dark cycle.
All procedures involving laboratory animal use were in accor-
dance with the guidelines of the Instituted Animal Care
and Use Committee of Chung Shan Medical University (IACUC,
CSMU) for the care and use of laboratory animals. The rats
were divided evenly into five groups of 8 rats and treated
intrapерitoneally with CCl4 (8% CCl4/corn oil, 1 mL/kg body
weight (BW) twice a week, Monday and Thursday) for 8
weeks, as described by Hernández-Muoz et al. [23], with
some modifications. At the same time, the rats were treated
with various dosages of OGE (0–0.04 g/kg BW), or silymarin
orally (0.2 g/kg BW, four times a week, Tuesday, Wednesday,
Friday, and Saturday) [24, 25]. The control rats were treated
with corn oil (1 mL/kg BW) and fed a normal diet. At the
end of the experiment, blood and heart were immediately
obtained after the animals were sacrificed.

2.3. Histological Examinations. The heart was fixed in 10%
formalin, processed using routine histology procedures,
embedded in paraffin, cut in 5 μm sections, and mounted on
a slide. The samples were stained with hematoxylin and eosin
for histopathological examination.

2.4. Preparation of Tissue Extract. All procedures were per-
formed at 4°C. The heart samples were lysed by 30 strokes
using a Kontes homogenizer at a ratio of 100 mg tissue/1
mL lysis buffer. The lysis buffer consisted of 50 mM Tris-
HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 150 mM NaCl,
1 mM PMSF, 10 μg/mL leupeptin, 1 mM sodium orthova-
date, 1% (v/v) 2-mercaptoethanol, 1% (v/v) Nonidet P40,
and 0.3% sodium deoxycholate. These homogenates were
centrifuged at 100,000 g for 1 h at 4°C. The supernatant
was stored at −70°C for Western blot assay.

2.5. Electrophoresis and Western Blot. Tissue extract sam-
ple were prepared as described above. Sodium dode-
cane sulfate-polyacrylamide gel electrophoresis is carried out as
described by Laemmli [26] using 10% polyacrylamide gels.
After samples are electrophoresed at 140 V for 3.5 h, the
gels are equilibrated for 15 min in 25 mM Tris-HCl, pH
8.3, containing 192 mM glycine and 20% (v/v) methanol.
Electrophoresed proteins are transferred to nitrocellulose
paper (Amersham, Hybond-C Extra Supported, 0.45 Micro)
using Hoefer Scientific Instruments Transphor Units at
100 mA for 1 h. The nitrocellulose paper was incubated
at room temperature for 2 h in blocking buffer containing
100 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v)
Tween 20, and 3% (v/v) fetal bovine serum. Antibodies
BNP, phospho-GATA binding protein 4 (p-GATA4), nuclear
factor of activated T cells (NFAT), mitogen-activated protein
kinase kinase 5 (MEK5), extracellular signal-regulated pro-
obtained after the animals were sacrificed.
3. Results

3.1. Changes in Heart Weight of CCl₄-Induced Cirrhosis-Associated Cardiac Hypertrophy. Throughout the experimental period of 8 weeks, there was no difference in body weight of rats within the 5 groups. At the end of the experiments when rat livers were measured, liver fibrosis was observed in the CCl₄-treated group, as compared to the control group which was given olive oil. And for the groups treated with OGE or silymarin, a protective effect was observed: liver fibrosis was significantly ameliorated compared to the CCl₄-treated group (data pending publication). In comparison, Table 1 shows that the whole heart weight (WHW), left ventricle weight (LVW), and their ratio to the body weight of the CCl₄-treated group were significantly higher than the control group. For groups treated with 0.02 g/kg BW OGE and treated with 0.2 g/kg BW silymarin, weights of the heart remained equal to the control group. However, for the group treated with 0.04 g/kg BW OGE, the weight values had a less significant decrease compared to the CCl₄-treated only group.

3.2. Changes in Diameter and Thickness and Histological Structure of Left Heart Ventricle of CCl₄-Induced Cirrhosis-Associated Cardiac Hypertrophy. The left ventricle diameter of the CCl₄-treated group was significantly larger and the walls were moderately thicker than the control group (Figure 1 upper panel and Table 2), but a change of that scale in ventricle diameter was not present in the OGE and silymarin cotreated groups.

The left most picture in Figure 1 (lower panel) shows the appearance of a normal heart: one with a unified tissue pattern. However, hearts treated with CCl₄ had clearly lost its tissue integrity, but such a change was clearly not observed in groups cotreated with 0.02 g/kg BW OGE and silymarin. The individual severity rates in rats were expressed as mean ± SE. BW: body weight; WHW: whole heart weight; LVW: left ventricle weight. * Significant differences from Group A, P < 0.05. # Significant differences from Group B, P < 0.05.

3.3. The Expression of Cardiac Hypertrophy Related Genes in the Heart of CCl₄-Treated Rats. The expression of cardiac hypertrophy related genes, such as BNP, p-GATA4, and NFAT4, were also tested [7]. Their figures were increased in the CCl₄-treated group as compared to the control group (Figures 2 and 3). In the groups cotreated with 0.02 g/kg BW OGE or silymarin, the expression of BNP, p-GATA4, and NFAT returned to control level. The results of the 0.04 g/kg BW OGE-treated group were consistent with the above figures, in that their expressions were decreased, but not back to control levels.

3.4. The Expression of IL-6 Signaling Pathway Related Genes in the Heart of CCl₄-Treated Rats. We wanted to test for IL-6 signaling pathways because studies have shown that cardiac hypertrophy can be attributed to IL-6 related cytokines [7]. Western blotting analysis shows that the expressions of IL-6, MEK5, ERK5, and p-ERK5 were increased in the CCl₄-treated group as compared to the control group.

Table 1: Changes in body weight and organ weight of CCl₄-induced cirrhosis-related cardiac hypertrophy.

|       | A*   | B   | C  | D  | E   |
|-------|------|-----|----|----|-----|
|       | (n = 8) | (n = 8) | (n = 8) | (n = 8) | (n = 8) |
| BW (g) | 425 ± 16.475 | 402 ± 8.920 | 385 ± 6.547 | 388 ± 10.823 | 420 ± 19.272 |
| WHW (g) | 1.041 ± 0.015 | 1.173 ± 0.031* | 0.975 ± 0.023f | 1.089 ± 0.026 | 1.023 ± 0.015f |
| LVW (g) | 0.813 ± 0.010 | 0.898 ± 0.018* | 0.745 ± 0.028f | 0.777 ± 0.021 | 0.767 ± 0.023f |
| WHW/BW (%) | 2.467 ± 0.095 | 2.918 ± 0.093* | 2.535 ± 0.065f | 2.813 ± 0.067 | 2.461 ± 0.101f |
| LVW/BW (%) | 1.922 ± 0.050 | 2.233 ± 0.045* | 1.933 ± 0.054f | 2.190 ± 0.062 | 1.844 ± 0.085f |
| LVW/WHW (%) | 0.781 ± 0.011 | 0.767 ± 0.012 | 0.764 ± 0.020 | 0.779 ± 0.015 | 0.751 ± 0.030 |

*Group A is given olive oil and water, Group B is given CCl₄ and water, Group C is given CCl₄ and 0.02 g/kg of OGE, Group D is given CCl₄ and 0.04 g/kg of OGE, and Group E is given CCl₄ and 0.2 g/kg of silymarin. The individual severity rates in rats were expressed as mean ± SE. BW: body weight; WHW: whole heart weight; LVW: left ventricle weight. * Significant differences from Group A, P < 0.05. # Significant differences from Group B, P < 0.05.

Table 2: Changes in diameter and thickness of left heart ventricle of CCl₄-induced cirrhosis-related cardiac hypertrophy.

|       | A*   | B   | C  | D  | E   |
|-------|------|-----|----|----|-----|
|       | (n = 8) | (n = 8) | (n = 8) | (n = 8) | (n = 8) |
| Diameter of LV (mm) | 8.17 ± 0.00 | 10.67 ± 0.22* | 8.50 ± 0.19f | 9.33 ± 0.22f | 8.83 ± 0.11f |
| Thickness of LV (mm) | 3.83 ± 0.11 | 4.43 ± 0.15* | 3.87 ± 0.12f | 4.17 ± 0.11 | 3.83 ± 0.11f |
| Thickness/diameter (mm) | 0.42 ± 0.01 | 0.42 ± 0.01 | 0.46 ± 0.02 | 0.45 ± 0.02 | 0.43 ± 0.01 |

*Group A is given olive oil and water, Group B is given CCl₄ and water, Group C is given CCl₄ and 0.02 g/kg of OGE, Group D is given CCl₄ and 0.04 g/kg of OGE, and Group E is given CCl₄ and 0.2 g/kg of silymarin. The individual severity rates in rats were expressed as mean ± SE. LV: left ventricle. * Significant differences from Group A, P < 0.05. # Significant differences from Group B, P < 0.05.

After washing with blocking buffer, the membrane was visualized using chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.6. Statistical Analysis. The experimental results are expressed as the mean ± SE. Data were assessed using analysis of variance (ANOVA) followed by a Student-Newman-Keuls correction to adjust the significance level to avoid a type I error. Student’s t-test was used in the comparison between groups. A P value less than 0.05 was considered statistically different.
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![Figure 1](image1.png)

Figure 1: Cardiac pathologic analysis in the heart of CCl₄-treated rats. Herbs and CCl₄ were given as described in Materials and Methods. The top panels show the heart of the macroscopic cross-section. The bottom panels show high magnification (×400) of tissue structure. LV: left heart ventricle; RV: right heart ventricle.

![Figure 2](image2.png)

Figure 2: The expressions of BNP by Western blotting analysis (a) and quantitative analysis (b) in the heart of CCl₄-treated rats. The individual severity rates in rats were expressed as mean ± SE, n = 8. *P < 0.05 as compared with control group. **P < 0.05 as compared with the CCl₄-treated group.

(Figure 4). In the groups cotreated with 0.02 g/kg BW OGE or silymarin, the expression of IL-6, MEK5, ERK5, and p-ERK5 returned to control level. The expressions were also lowered in the 0.04 g/kg BW OGE-treated group, but not back to the levels of the control group.

The expressions of other IL-6 signaling pathway genes, p-JAK and STAT3, were tested, the data shows that both their expressions were increased in the CCl₄-treated group as compared to the control group (Figure 5). In the groups cotreated with 0.02 g/kg BW OGE or silymarin, the expressions of p-JAK and STAT3 returned to control levels, except for the 0.04 g/kg BW OGE group, which were lowered but not back to the control levels. This result is consistent with the data above.
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120 kDa
57 kDa
92 kDa-p-GATA4
NFAT3
α-tubulin

(a)

Control
CCl4
CCl4+ 0.02 g/kg OGE
CCl4+ 0.04 g/kg OGE
CCl4+ 0.02 g/kg silymarin

Figure 3: The expressions of NFAT3 and phosphorylated-GATA4 by Western blotting analysis (a) and quantitative analysis (b) in the heart of CCl4-treated rats. The individual severity rates in rats were expressed as mean ± SE, n = 8, * P < 0.05 as compared with control group, and # P < 0.05 as compared with CCl4-treated group.

4. Discussion

Numerous reports center on the involvement of IL-6 and the related cytokines in cardiac hypertrophy [7] as an inducer of downstream pathways. IL-6 is a typical cytokine which was found to have a potent hypertrophic effect on cardiomyocytes [27], as the overexpression of this cytokine has been linked to hypertrophic myocardium injury [28]. In the present study, our data showed that the expressions of IL-6 increased in CCl4-induced cirrhosis rats detected with occurrence of cardiac hypertrophy, which suggests that the cirrhosis-associated cardiac hypertrophy may be related with the IL-6 signaling pathway in the CCl4-treated rats.

IL-6 is involved in multiple intracellular signaling pathways, particularly the MEK5-ERK5 pathway [29–32], which plays a critical role in the induction of eccentric cardiac hypertrophy that can progress to dilated cardiomyopathy and sudden death [33, 34], and the JAK-STAT3 pathway, which promotes the increase of cell dimensions [35–37]. Since the experiments suggest a relationship between CCl4-induced cirrhosis-associated cardiac hypertrophy and IL-6, we decided to analyze the mechanism concerning OGE and silymarin and how it may inhibit cardiac hypertrophy through the inhibition of IL-6 extracellular signals. Western blotting analysis shows that the expressions of IL-6, MEK5, ERK5, and p-ERK5 were increased in the CCl4-treated groups as compared to the control (Figure 4) and were partially restored to control levels when cotreated with OGE or silymarin. Moreover, the expressions of p-JAK and STAT3 were increased in the CCl4-treated group (Figure 5) and restored by OGE or silymarin cotreatment, as in the above gene expressions. Taken together, these findings indicate that both the JAK-STAT3 and the MEK5-ERK5 pathways related genes were overexpressed by IL-6 expression in response to CCl4-induced cirrhosis-associated cardiac hypertrophy (Figure 6), which confirms the importance of the two pathways and also demonstrates that their overexpression may be reversed by OGE or silymarin treatment thus lowering liver cirrhosis and reducing the chance of cardiac hypertrophy. An interesting note is that silymarin, which has rarely been
Figure 4: The expressions of IL6 and its downstream signaling proteins MEK5, ERK5, and phosphorylated-ERK5 by Western blotting analysis (a) and quantitative analysis (b) in the heart of CCl4-treated rats. The individual severity rates in rats were expressed as mean ± SE, n = 8, *P < 0.05 as compared with control group, and #P < 0.05 as compared with CCl4-treated group.
Figure 5: The expressions of JAK-Stat3 pathway by Western blotting analysis (a) and quantitative analysis (b) in the heart of CCl4-treated rats. The individual severity rates in rats were expressed as mean ± SE, n = 8. *P < 0.05 as compared with control group. #P < 0.05 as compared with CCl4-treated group.

Figure 6: The summary of the mechanism of CCl4-induced cirrhosis-associated cardiac hypertrophy. Our data demonstrated that Ocimum gratissimum and silymarin extracts attenuate cardiac cells from CCl4-induced damage possibly by lowering liver cirrhosis which reduces the chance of cardiac hypertrophy maybe via inhibiting IL-6 signaling pathway activation.
demonstrated to treat cardiac hypertrophy [38], suggests that some common elements between herbal preparations, such as their antioxidant properties, may be responsible for treatment against liver cirrhosis-induced cardiac damage.

Cardiac hypertrophy can be classified as physiological and pathological hypertrophy [7], with the physiological being a natural bodily response to maturation, pregnancy, and exercise, and the pathological being a response to pathological stress signals, such as inflammation, cardiac injury, or exposure to toxicity. In our study, we found that many genes were responded to cardiac hypertrophy by CCl4 induction, including MEK5, ERK5, JAK2, STAT3, NFAT3, GATA4, and fetal gene BNP, which are used as a pathological marker [39–42] (Figure 6). Since pathological hypertrophy is also associated with observable loss of tissue integrity, which we also found in CCl4-treated rats, this suggests that CCl4 induced cirrhosis-associated cardiac hypertrophy may belong to pathological hypertrophy and can also be explored further as a pathological model.

There is a peculiar phenomenon that a 0.02 g/kg BW dose of OGE had a significant inhibition effect on CCl4-induced cardiac hypertrophy and on the related gene expressions than a 0.04 g/kg BW dose. A possible explanation suggests that the saturation of the higher dose could have lowered the effectiveness of the treatment.

5. Conclusions

In summary, CCl4-induced cirrhotic cardiac damage can occur through the IL-6 signaling pathway which leads to eventual cardiac hypertrophy. OGE and silymarin can protect cardiac cells from CCl4-induced damage possibly by inhibiting the expression of the IL-6 signaling pathway related genes. Moreover, we also found in further research that CCl4 induced cardiac damage can induce the FASL signaling pathway and the TGF-β signaling pathway, which may lead to cell apoptosis and eventual cardiac fibrosis (pending publication). It seems that multiple mechanisms are involved in the CCl4 induced cardiac damage. However, in the present study, we suggest that OGE and silymarin in the form of herbal supplements are a viable option for the protection of cardiac tissues against cirrhosis-related cardiac hypertrophy.

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