

Protective Effects of GUF (*Glycyrrhiza uralensis* Fischer) Extract in Water Immersion Restrain Stress (WIRS) Induced Gastric injury Rat Model

Jun-Soon Lee, Kyung-Yae Hyun*

¹ Department of Clinical Laboratory Science, Dong-Eui University, Busan 614-714, Korea

Abstract

In this paper purpose is investigate protective effects of GUF (*Glycyrrhiza uralensis* Fischer) Extract in Water Immersion Restrain Stress (WIRS) induced Gastric injury rat model. 30 min after GUF orally administered in rat, implemented water immersion during 8 hr. studying animal groups are as follows; Group I : Control group(n=5), group II : WIRS induced Rat, Group III: WIRS induced + GUF Extract 25mg/kg treated Rat, Group IV: WIRS induced + GUF Extract 50mg/kg treated Rat, Group V: WIRS induced + GUF Extract 100mg/kg treated Rat. We confirmed gastric injury through stomachs were opened along the greater curvature and gastric lesion area was quantified by pixel density using digital camera and we experimented western blotting for investigate inflammation related signal transduction protein expression differences in Liver. Researcher result showed oral administration of GUF Extracts has dose dependent protective effects in gastric injury and suggested having improvement effect of inflammation.

Keywords: *Glycyrrhiza uralensis* Fischer, Water Immersion Restrain Stress, Gastric injury, NF-kB, iNOS

1. Introduction

Gastric injury is one of the typical diseases mainly caused by mental-physical stressors. It may be caused inevitably by increased physical stress due to alcohol intake or smoking along with mental stress in increasingly more segmented and complicated society of today.

The effects of stress on the mental-physical balance may induce multiple pathological changes. Gastric injury, one of the pathological changes induced by stress, has several causes: inhibition of gastric mucous functions due to inhibited prostaglandin synthesis in gastric mucosa and changed excretion of gastric acid, mucus, and bicarbonate, reduced blood flow in gastric mucosa, changed stress hormone, and so on. These pathological changes may result in gastric injury [1]. They may also cause abnormal heart beat and increase the respiration rate due to stress and make trouble in the heart and especially in the liver. The incidence rate of gastric ulcer in South Korea ranges from 6% to 15% and everyone is expected to suffer the condition at least more than once. Despite such a high incidence rate, it is an incurable disease, which cannot be

cured completely; for this reason, research in natural substances with no adverse effect is being conducted positively [2].

Glycyrrhiza uralensis Fischer is a perennial herbaceous plant, which belongs to the bean family, and is pharmacognosy; some roots and stalks of the same species plants are used with or without peel. As can be known from the words, "*Glycyrrhiza uralensis* Fischer in a drugstore," it is primarily used in preparation of herb medicines: it is used for about 90% of all the preparations in the Oriental medicine [3]. The principal component of *Glycyrrhiza uralensis* Fischer, glycyrrhizin, gives it a sweet taste. It is effective in protecting the liver [4], ion preventing fat accumulation and apoptosis [5], in preventing pancreatitis [6], in glycemic control [7], and in anti-inflammatory functions [8].

Some of the typical animal models for stress-caused gastric injury are Water Immersion Restrain Stress (WIRS) models, indomethacin induction models, and ethanol induction models [9]. WIRS, which is an animal model for causing stress through fear of water and behavioral restriction, has been used in the research on excretion of gastric acid in gastric ulcer or on the roles of resultant necrosis [10]. We used this model to determine the efficacy of *Glycyrrhiza uralensis* Fischer extracts on gastric injury caused by severe stress. To do this, we personally incised the stomach, observed the injured region with naked eye, took pictures of the stomach with a digital camera, qualified the pixel density in the injured region, made a comparative analysis of the expression levels of NF-kB, NOX4, iNOS, and Nrf2, which are signal transfer proteins related to inflammation and anti-oxidation in the liver expected to be injured by the stress, and determined the variation in the physiological defense mechanism due to stress and whether *Glycyrrhiza uralensis* Fischer protected the stomach from being injured by stress.

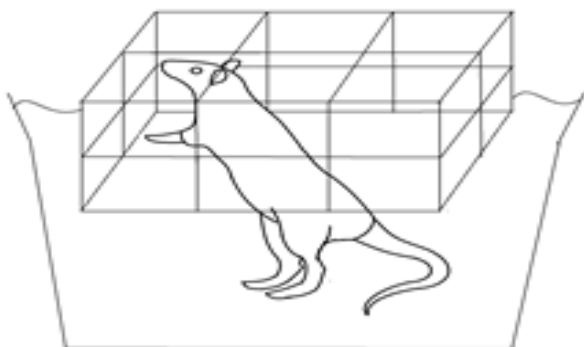
2. Method

2.1 Test animals and animal grouping

25 Sprague-Dawley 6-week male white rats (150-170 g) were supplied by Oriental Bio. Co. The rearing environment involved temperature at 25°C, humidity at 60%, and a 12-hour day-night cycle and they were free to have the same amount of feed and water. All experiments were approved by the Ethics Committee of Dong-Eui University and were in accordance with the guidelines of the International Association for the Study of Pain (IASP). The rats were classified into Group I with oral administration of saline solution (oral group) (control group, n=5), Group II with 8 hours of WIRS, Group III with 8 hours of WIRS and oral administration of *Glycyrrhiza uralensis Fischer* (25 mg/kg(rat weight)) (n=5), Group IV with 8 hours of WIRS and oral administration of *Glycyrrhiza uralensis Fischer* (50 mg/kg(rat weight, n=5), and Group V with 8 hours of WIRS and oral administration of *Glycyrrhiza uralensis Fischer* (100 mg/kg(rat weight, n=5). Oral administration was divided into low, middle, and high concentration.

2.2 Experimental model

The rats were not fed for 24 hours before the experiment and were orally administered with 25 mg/kg, 50 mg/kg, and 100 mg/kg according to the concentration of *Glycyrrhiza uralensis Fischer* extracts 30 minutes before being sacrificed. 30 minutes after the oral administration, they were dipped in 22°C water for 8 hours and were put in a cage to control their behavior. They were sacrificed immediately 8 hours later to proceed with the subsequent experiments. The experiment modeling was shown in Figure 1.



[Fig. 1] Water Immersion Restrain Stress (WIRS) Rat model 30 min after GUF (25mg/kg, 50mg/kg, 100mg/kg) orally administered, Rats are immersed in water (22°C) during 8 hr. Rat behavior are limited by restraint cage.

2.3 Analysis Methods

2.3.1 Measurement of Gastric Damage Lesion

We administered 20 ml saline solution through the esophagus to the rats that had been sacrificed immediately after being dipped in water for 8 hours to swell their stomach; then, we tied the lower esophageal sphincter and pyloric sphincter with thread and cut them to incise the gastric large curve, washed it with saline solution, spread and fixed the stomach, took its pictures with a digital camera, quantified pixel density in the gastric region injured with ulcer, and made quantitative analysis. The photos were shown in Figure 2.

2.3.1 Measurement of Gastric Damage Lesion

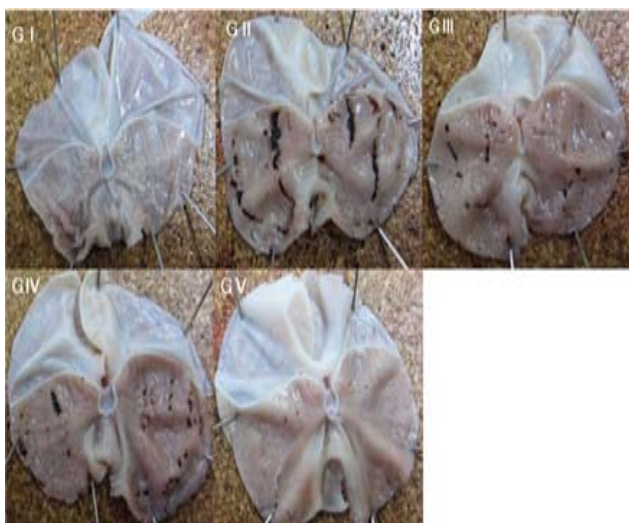
We extracted the liver from the sacrificed rats and used a surgical knife to cut it into small pieces. We added lysis buffer (PRO-Prep™, protein extraction solution) to the tissues and used a homogenizer (Intron Biotechnology, Gyeonggi-do, Korea) to homogenize them. We centrifuged the homogenized tissues for 15 minutes at 13000 RPM and 4°C to separate foreign matters from the supernatant, diluted the supernatant into a proper concentration, used the Bio-Rad Protein assay Kit (Bio-Rad Hercules, CA, USA) to develop color, and used an X-Mas Spectrophotometer (Human Cor. Korea) for measurement and preparation in the absorbance scope from 0.4 to 1 at 595 nm. A sample buffer was mixed with each supernatant and quantified to make the protein concentration of all the samples identical. We heated each sample at 95°C for 10 minutes, gave it electrophoresis at 70 V for 30 minutes, at 100 V for an hour and half, and at 110 V for an hour and half in acrylamide gel and transferred protein to the nitrocellulose membranes over an hour and half. We used a blocking buffer (5% Skim milk + PBST) to block them twice for an hour each time and diluted a proper volume of primary antibodies (NF-κB, NOX4, iNOS, Nrf2) to make them react to refrigeration for 24 hours. After the reaction, we washed them, made the secondary antibodies (rabbit or mouse) react for 2 hours, washed them again, and used ECL prime (Amersham Pharmacia Biotech, Buckinghamshire, UK) to analyze protein expression in a darkroom.

2.3.3 Statistical processing

We used a digital camera to take pictures of gastric ulcer and injury lesions and measured the longest diameter in each gastric ulcer lesion to synthesize, average, and quantify them in each group. The quantification was analyzed through ANOVA using SPSS Version 18. The findings from the quantitative analysis of protein were analyzed using Vision Works Image Software.

3. Results

3.1 protective effects of *Glycyrrhiza Uralensis Fischer* extracts against WIRS-induced gastric injury. The visually identified levels of gastric ulcer and injury are shown in Figure 2. Gastric injury was more remarkable in Group II than in Group I even visually; in particular, Group II (vehicle group) had the highest level of injury along with bleeding and the level of gastric injury was lowered in a concentration-dependent way in Groups III, IV, and V that were orally administered with *Glycyrrhiza uralensis Fischer*.

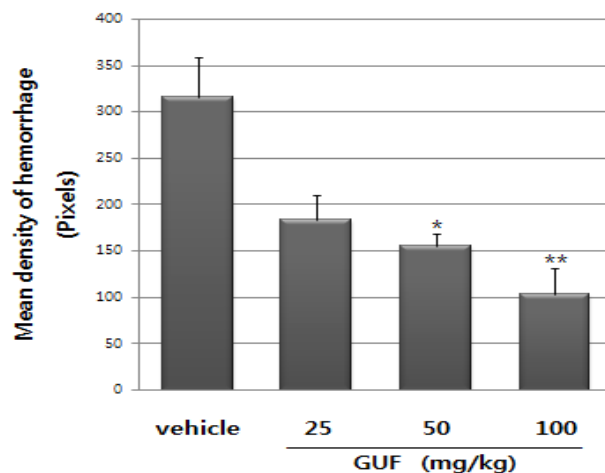


[Fig. 2] protective effects of GUF in WIRS induced gastric injury. These pictures showed protective effects of GUF in WIRS induced gastric injury in rat and protective effects are dose dependently increase. G□: Control Group, G□: Vehicle Group, G□: 25mg/kg GUF, G□: 50mg/kg GUF, G□: 100mg/kg GUF.

3.2 Pixel density quantification in regions with gastric injury

The graphs for quantified levels of gastric ulcer and injury in micro-pixel density are shown in Figure 3. We

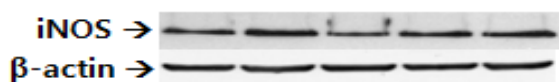
used a digital camera to take pictures of the stomach, summed up all the measurements of the longest diameter in each lesion size, and compared the sum in each group [11]. On the basis of visual observation, Group II (vehicle group) had a very high level of density due to the greatest injury, tended to decrease according to the concentration of *Glycyrrhiza uralensis Fischer* extracts, and decreased significantly at 50 mg/kg and 100 mg/kg ($p < 0.05$, $p < 0.01$).



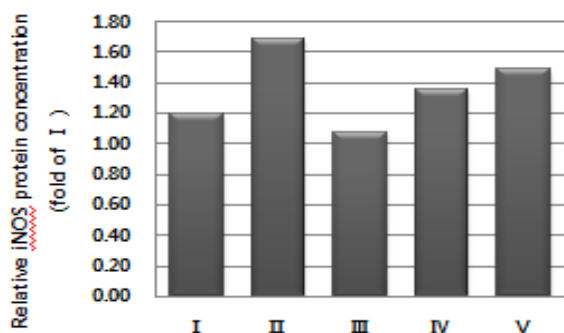
[Fig. 3] Quantification of gastric injury lesion area by pixel density. *, $p < 0.05$ (compare with vehicle); **, $p < 0.01$ (compare with vehicle). The gastric injury lesion size was quantified by measuring each lesion along its greatest diameter. Each groups of total fixel density expressed and averaged as the lesion index.

3.3 Quantitative analysis of protein

The levels of iNOS, NOX4, NF-kB, and Nrf2 expression in the liver are shown in Figure 4, Figure 5, Figure 6, Figure 7. All of the four signaling proteins had higher levels of protein expression in Group II than in Group I and were at remarkably lower levels in all of Groups III, IV, and V than in Group II though visual observation found no decrease in gastric injury according to the concentration of *Glycyrrhiza uralensis Fischer* extracts.



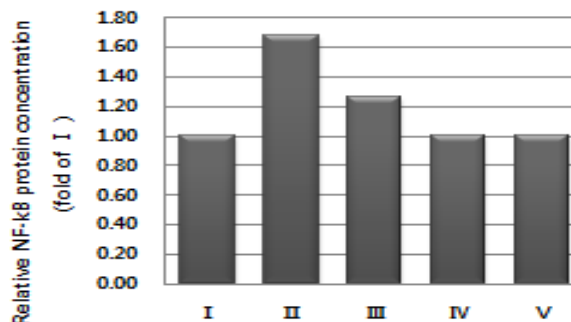
iNOS in Liver



[Fig. 4] iNOS protein Expression in the Liver. I . Control; II. WIRS induced Rat; III, IV, IV WIRS + Ginseng leaf Extract 200,100,50mg/kg treated Rat.



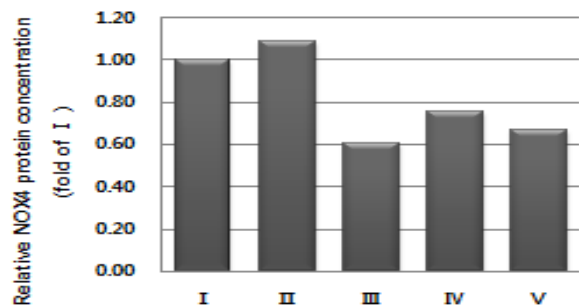
NF-kB in Liver



[Fig. 6] NF-kB protein Expression in the Liver. I . Control; II. WIRS induced Rat; III, IV, IV WIRS + Ginseng leaf Extract 200,100,50mg/kg treated Rat.



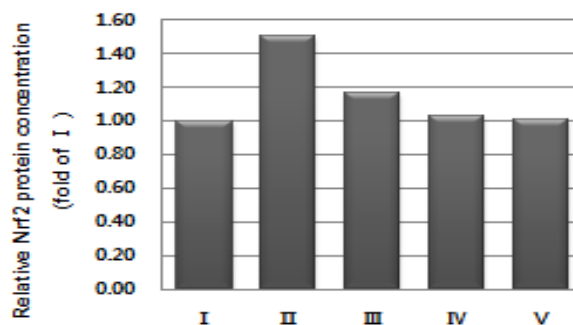
NOX4 in Liver



[Fig. 5] NOX4 protein Expression in the Liver. I . Control; II. WIRS induced Rat; III, IV, IV WIRS + Ginseng leaf Extract 200,100,50mg/kg treated Rat.



Nrf2 in Liver



[Fig. 7] Nrf2 protein Expression in the Liver. I . Control; II. WIRS induced Rat; III, IV, IV WIRS + Ginseng leaf Extract 200,100,50mg/kg treated Rat.

4. Discussion

Gastric diseases are very common conditions that affect millions of people each year; in particular, gastric ulcer causes local defects in the stomach, inhibits preservation of gastric mucosa, and may make perforation due to inflammation. The aggressive and defensive factors of gastric injury are now known to play a crucial role in gastric injury. The aggressive factors include gastric acid, pepsin, bile reflux, non-steroidal anti-inflammatory drugs (NSAIDs), helicobacter pylori, and alcohol and the defensive factors include mucous blood flow, epithelial cells, prostaglandin, mucus, and gastric exercise [12]. Gastric injury may be caused by the balance between the

aggressive and defensive factors; in particular, the decreased number of defensive factors reportedly had greater effects than the increased number of aggressive factors. This is because aggressive factors may attack the regenerated mucosa again after the discontinuation of administration with its inhibitor; thus, it is more effective to protect mucosa from the aggressive factors by promoting the defensive factors [13]. Lots of medicines currently used for gastric ulcer have therapeutic effects but may cause various problems, for example, due to tolerance. So we performed an experiment to determine the therapeutic effects of the natural substances, *Glycyrrhiza uralensis Fischer* extracts, against gastric injury and the possibility of substituting them for the current medicines. The gastric large curve has been incised and spread to identify lesions in a standardized way in an attempt to determine the efficacy of drugs against gastric injury and we used such a method to make observation [14, 15, 16]. We identified gastric injury lesions with naked eye, took their pictures with a digital camera, and made a quantitative comparison of gastric injury levels in pixel density. The visual observation found the concentration-dependent effectiveness in protecting gastric mucosa, so did the graph with quantified pixel density, showing significant effects at 50 mg/kg and 100 mg/kg. This result demonstrates that the *Glycyrrhiza uralensis Fischer* extracts are effective in protecting gastric mucosa by controlling the balance between the aggressive and defensive factors. It is well known that stress is associated with ulcer in digestive organs and circulation disorders. This fact implies that stress is associated with cancer progression, with immunosuppression confirmed by animal test. Intermittent, long-term mental stress may cause oxidative stress in nuclear DNA of the liver; the OH8dGuo level, as an injury index of nuclear DNA, rose significantly with long-term, repetitive stress; and failure to restore damaged DNA may reportedly cause point mutation [17]. Damaged liver functions due to such stress may inhibit preservation of mucous protection factors, thus causing gastric injury. Nrf2 may reportedly be activated by reactive oxygen species (ROS) and mitogen activated protein kinase (MAPK), enter nucleus, induce antioxidant responsive element (ARE) expression, perform anti-oxidative functions, and serve to protect cells in several organs [18]. The research with the improvement in the model of gastric ulcer induced by WIRS of Gorosoe water found that isoforms of NOS, such as eNOS and iNOS, played an important role in maintaining the functions through blood flow in gastric mucous cells, homeostatic blood vessels, and inflammation control and the Gorosoe water administration group had mRNA expression of iNOS significantly decreased [19]. In the model of diabetes induced by oxidative stress, 7-O-galloyl-D-sedoheptulose (GS) may reportedly control oxidative stress,

inflammation, and apoptosis in the liver and improve the condition; NOX4, a subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase involved in ROS generation, Nrf2 and HO-1, signal transfer proteins related to anti-oxidative stress, and such signal transfer proteins related to inflammation as NF-kB, iNOS, and COX2 were compared in terms of the expression level [20]. The factors known to affect signal transfer activity of NF-kB directly or indirectly are proinflammatory cytokines, such as IL-1 β and TNF- α , and reactive oxygens intermediates (ROIs). They may activate the signal transfer system through TLR and TNF receptors and ultimately phosphorylate p65 combined with inhibitor κ B within cytoplasm and make it enter nucleus [21]. Although the concentration of cytokines in blood was not measured in this experiment, the decrease in protein expression of NF-kB in the *Glycyrrhiza uralensis Fischer* administration group demonstrates the decreased concentration of inflammatory cytokines in blood. Literature review found that NF-kB, NOX4, iNOS, and Nrf2 expression in the liver was all at lower levels than in Group II. On the basis of these findings, administration with *Glycyrrhiza uralensis Fischer* extracts may serve as a NF κ -B phosphorylation inhibitor in a situation with severe, long-term stress [22] and be effective in protecting the stomach from being injured by stress by promoting defensive factors through anti-inflammation and anti-oxidation. *Glycyrrhiza uralensis Fischer* is expected to become a candidate for gastritis therapy.

5. Conclusion

When sees all things considered, we produce fresh observation that oral administration of GUF may have preclusive and therapeutic effects on gastric injury induced by stress or emotional stress. Our study also has demonstrated the stomach protective and anti-inflammatory effects of GUF extracts from water immersion restraint stress rat model, which would be correlated to the control of significant signaling pathways such as MAK kinase and NF-kB controlling the production of inflammatory mediators.

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