



ANTI-INFLAMMATORY EFFECTS OF HIPPOPHAE RHAMNOIDES AND BOSWELLIA EXTRACTS ON MASSETER MUSCLE AND GASTROCNEMIUS INFLAMMATION IN RATS

Hae-Jin Jeong¹, Byeong-Jo Kim¹ and Tae-Heon Kim^{2*}

¹Dept. of Biomedical Health Science, Dong Eui University.

²Dept. of Dental Hygiene, Taegu Science University.

*Corresponding Author: Dr. Tae-Heon Kim

Dept. of Dental Hygiene, Taegu Science University.

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ABSTRACT

This study aimed to establish an inflammatory model by injecting carrageenan into masseter muscle and gastrocnemius muscle in rats, and then verify the anti-inflammatory effects of both Hippophae rhamnoides and Boswellia extracts. As experimental animals, male Sprague-Dawley rats aged 7-8 weeks (250-300 g) were used. The inflammation of masseter muscle and gastrocnemius was induced by injecting 0.3 mL of 2% carrageenan diluted with normal saline solution into them. Hippophae rhamnoides and Boswellia extracts were orally administered into the rats, 30 minutes before the inflammatory induction. In order to verify changes in tissues, masseter muscle and gastrocnemius muscle were extracted, 4 hours after the injection of the carrageenan. The tissues were photographed by using an optical microscope, after slicing and staining of them, and the area of cells was measured by using Image J (1.52a, USA). For the quantitative evaluation of measured cell changes, SPSS statistic ver. 22 was used to conduct a statistical analysis. The results of this experiment show the significant dose-dependent increase in the area of cells of most rats in the extract administration group, compared to the carrageenan group. The sizes of muscle cells in masseter muscle, especially Bos30 group and Bos15+Vit150 group, were significantly increased, depending on the concentrations of both vitamin tree and Boswellia extracts. For the gastrocnemius muscle, anti-inflammatory effects were induced in the extract administration group, compared to the control group, but the effects were not significant. From the results, the effects of Hippophae rhamnoides and Boswellia on the inflammatory regulation of masseter muscle and gastrocnemius could be proved, suggesting that natural substances can be effective drug for the inflammatory regulation of muscle.

KEYWORD: Hippophae rhamnoides, Boswellia, Inflammation, Masseter Muscle, Gastrocnemius.

INTRODUCTION

The stability of treatments for clinical symptoms has been always an emerging issue. The treatments for muscle injury substantially influencing patients' daily activities approach curing them in many ways. By using a variety of methods including physical therapy, injection therapy, electric needle therapy, etc. the treatments make an approach to reducing pain of patients and curing diseases. Although such methods may result in positive effects, they also have many side-effects. Injection and electric needle therapy induce the contagion caused by needles and fear in patients, and drug therapy may cause gastrointestinal disorders. Various measured have been tried to reduce such side-effects. Among them, alternative therapies using many natural substances increasingly take much attention.

Various studies have been recently conducted for natural substances. Among those whose pharmacological efficacy is proved, Hippophae rhamnoides (Hippophae

rhamnoides L., sea buckthorn) belonging to the family of *Ficus religiosa* contains the rich content of vitamin C, D and E, is called the vitamin tree (Choi et al., 2016) and highlighted. The methanol extracts of seeds from Hippophae rhamnoides are reported to have anti-inflammatory effects on *Bacillus cereus*, *Bacillus coagulans*, *Bacillus subtilis*, etc (Negi et al., 2005; Park et al., 2010a). In addition, the report (Jiang et al., 2017; Park et al., 2010b) showing that the Hippophae rhamnoides leaf extracts suppress the expression of nitric oxide (NO), interleukin 6, tumor necrosis factor- α , inducible nitric oxide synthase, and cyclooxygenase-2 proteins, caused by LPS, by inhibiting the activation of NF- κ B and the phosphorylation of p38 mitogen-activated protein kinase (MAPK) implies the antioxidant and anti-inflammatory effects of Hippophae rhamnoides. Meanwhile, Boswellia (*Boswellia Serrata*) which has been known to affect on arthritis, through many media and cases grow wild in the Red Sea, the whole northeastern Africa and produce myrrh (Leung and

Foster, 1996). Its representative substances, Boswellic Acid and Terpenoid are reported to have antiphlogistic and analgesic effects (Sailer et al., 1996). Nam et al. (2014) reported that the effects of *Boswellia* extracts on osteoarthritis of animal experiment model, which was induced by cultured cartilage cells and MIA were examined to show that the expression of NF κ B, cytokine IL-6, TNF- α was reduced.

Muscle injury and inflammation induce some physical functional disorders caused by flare, edema, pain, etc. as physical defense mechanism against physical impact, chemical materials and bacterial infection (Jew et al., 2003), and physical malfunction has been reported to have an effect on the reduction of muscle mass, as well as the occurrence of metabolic and degenerative diseases (Beaufre and Morio, 2000). According to previous studies, there are differences in nerve domination and positions, between facial and low limb muscle dominated by trigeminal and spinal nerves, and their differences in effects of psychotropic medicines are also exhibited (Bagüés et al., 2017, Kim et al., 2015). Although there have been many studies demonstrating the effects of natural substances applied to a specific local site, few studies have verified the effects of a single medicine injected on inflammatory regulation of muscle at different positions.

This study thus administered *Hippophae rhamnoides* and *Boswellia*, the representative natural substances which have been known to be effective in regulating inflammation and attempted to observe the differences in the effects of the natural substances on changes in cells, in regulating the inflammation of masseter muscle and gastrocnemius, which is induced by carrageenan

MATERIAL AND METHOD

Experiment Animals

Sprague-Dawley rats aged 7-8 weeks (250-300 g), which were bought from Hyochang Science (Daegu, Korea) were used as experiment animals. Feed and water were freely provided to them, while maintaining the constant temperature (23~24 °C) and alternatively changing the lightening according to day and night, every 12 hours. This experimentation observed the ethics about the experiment on animals, prescribed by the Korean Pain Research Society.

Drug Injection

15 mg/kg (Bos15) and 30 mg/kg (Bos30) of *Boswellia* extract (Bioprex Labs Co., Ltd., Pune Maharashtra, India), 150 mg/kg (HR150) and 300 mg/kg (HR300) of *Hippophae rhamnoides* extract (Shanghai Brichtolinternatinal Co., Ltd., Tibet, China) and 1 ml of mixture (Bos15+HR150) of 15 mg/kg of the former and 150 mg/kg of the latter were orally administered, after diluting it with distilled water. Then, 2% carrageenan diluted with saline was injected into the masseter muscle and gastrocnemius of rats, 30 minutes after the oral administration.

Preparation of Tissues

The rats were anesthetized with 20% urethane (0.5 ml/kg) and sacrificed after 4 hours after 2% carrageenan were injected. And then the muscles dissected. The extracted tissues were fixed in 10% formalin solution and then the tissues were made paraffin block. 5 μ m-thick sections were cut and stained with hematoxylin & eosin. The slides were observed by using an optical camera (CX22LEDRFS, Olympus co., Tokyo, Japan) and photographed with a digital camera and then, saved as a JPG file.

Quantitative and Statistical Analysis

Each of the experimental groups randomly selected five photos and the area of cell was measured by using Image J (1.52a, USA). A statistical analysis on the size of measured cells was conducted by using SPSS statistic ver. 22 (IBM Co., Armonk, NY, USA) ($p < 0.05$).

RESULT

Morphological Change

Although most of masseter muscle cells in the naive group had the forms of polygons and were densely concentrated, as there were very little spaces among the cells, while the majority of 2% carrageenan group had the forms of spheres, and there were spaces among the cells more than naive group. In addition, many polygonal cells were observed more than sphere cells in the Bos and HR group (Fig 1.).

The forms of gastrocnemius muscle cells were similar with those of masseter muscle cells (Fig 2). A majority of gastrocnemius muscle cells in the naive group were observed to have the forms of spheres, and the gastrocnemius muscle cells in the Bos and HR group relatively often had the forms of polygons. There were very little spaces among the cells in the naive group, while the majority of the 2% carrageenan group had the forms of spheres, and there were spaces that were wider than the former, among the cells in the 2% carrageenan group. The spaces among cells in the Bos and HR group were observed to be relatively narrower than those to which 2% carrageenan group.

Quantitative Analysis

The areas of masseter muscle cells in naive group, 2% carrageenan group, Bos15 group, Bos30 group, HR150 group, HR300 group and Bos15+HR150 group were 92730.26 ± 16461.26 , 48267.90 ± 14894.90 , 57645.00 ± 11142.48 , 75562.00 ± 14259.48 , 70113.64 ± 15026.13 , 73952.37 ± 30078.79 and 73497.98 ± 10850.51 , respectively. The area of cells in the naive group was narrower than 2% carrageenan group, while the areas of cells in the remaining groups (Bos15, Bos30, HR150, HR300, and Bos15+HR150) were wider than 2% carrageenan group.

The areas of gastrocnemius muscle cells in naive group, 2% carrageenan group, Bos15 group, Bos30 group, HR150 group, HR300 group and Bos15+HR150 group

were 83984.90 ± 24151.93 , 64799.26 ± 19941.74 , 79529.87 ± 24915.95 , 81072.40 ± 26448.35 , 75952.54 ± 21251.73 , 72930.12 ± 17927.24 , and 79263.40 ± 24487.19 , respectively. Similar to the aspects of masseter muscle, the area of gastrocnemius muscle cells in naive group was narrower than 2% carrageenan group, while the areas of cells in the remaining groups (Bos15, Bos30, HR150, HR300, Bos15+HR150) were wider than 2% carrageenan group. In contrast to the result of the masseter muscle, the area of cells in HR300 group was observed to be narrower than HR150 group.

Statistical Analysis

Compared to the area of rats' masseter muscle cells in the 2% carrageenan group, those in Bos15 group, Bos30 group, HR150 group, HR300 group, Bos15+HR150 group were more significantly increased. The area of the muscle cells in Bos30 group was more significantly increased than Bos15 group ($p < 0.05$) and Bos15+HR150 group was significantly increased than Bos15 group and HR150 group ($p < 0.05$). There were, however, no statistically significant differences in the areas, among Bos15+HR150, Bos 30, HR300 group.

Compared to the area of rats' gastrocnemius muscle cells in the 2% carrageenan group, Bos15 group, Bos30 group, HR150 group, HR300 group, Bos15+HR150 group were also more significantly increased. There were, however, no significant differences in the area of the muscle cells among Bos15, Bos30, HR150, HR300 group. In addition, there were no statistically significant differences in the area of the muscle cells among Bos15+HR150, Bos15, Bos30, HR150, HR300 group.

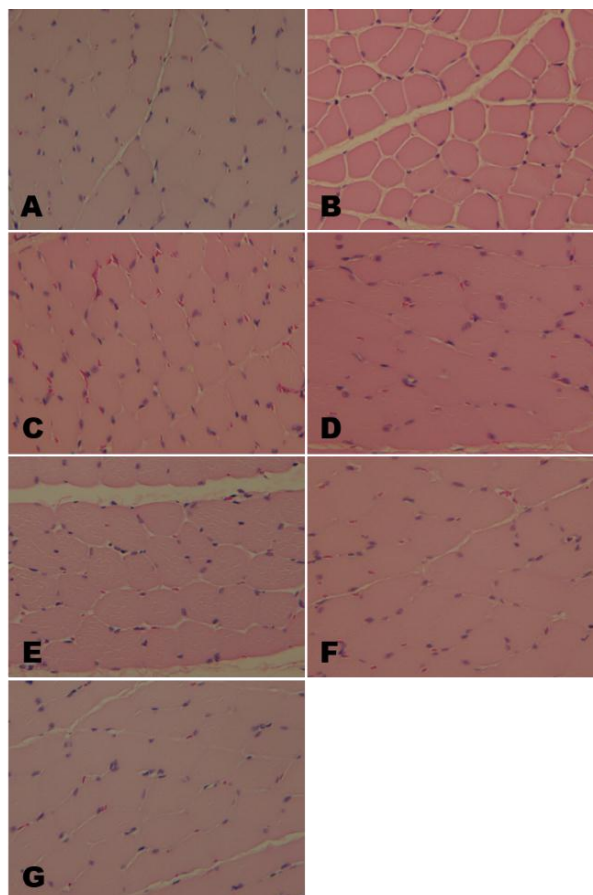


Fig 1: Anti-Inflammatory Effect of Hippophae rhamnoides and Boswellia Extracts in rat's Masseter Muscle. (Magnification: x400) A: Naive group, B: 2% carrageenan injecting group, C: 15 mg/kg of Boswellia extract administering group, D: 30 mg/kg of Boswellia extract administering group, E: 150 mg/kg of Hippophae rhamnoides extract administering group, F: 300 mg/kg of Hippophae rhamnoides extract administering group, G: mixture (15 mg/kg of Boswellia extract and 150 mg/kg of Hippophae rhamnoides extract) administering group.

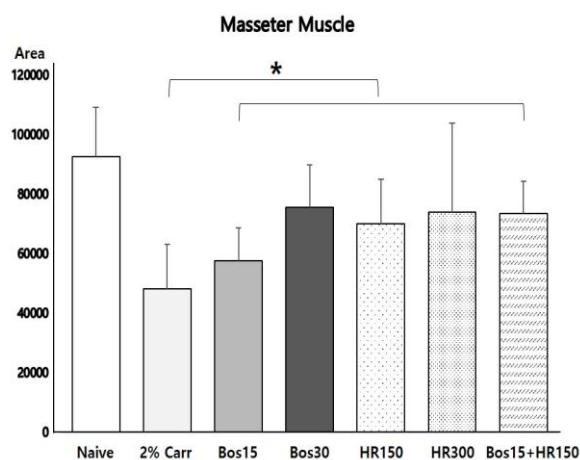


Fig 2: Area of cell in rat's masseter muscle. Boswellia and Hippophae rhamnoides Extracts administering groups increased cellular area more than 2% carrageenan injecting group. The cell area tended to

increase with the concentration of the administrated extract. 2% carr: 2% carrageenan injecting group, Bos15: 15 mg/kg of Boswellia extract administering group, Bos 30: 30 mg/kg of Boswellia extract administering group, HR150: 150 mg/kg of Hippophae rhamnoides extract administering group, HR300: 300 mg/kg of Hippophae rhamnoides extract administering group, Bos15+HR150: mixture of 15 mg/kg of Boswellia extract and 150 mg/kg of Hippophae rhamnoides extract administering group. * $p < 0.05$

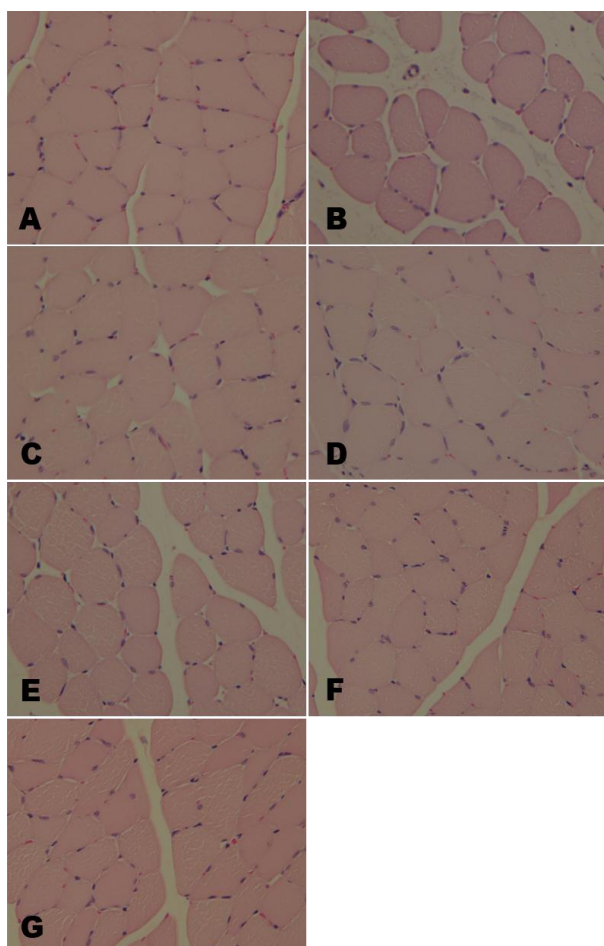


Fig 3. Anti-Inflammatory Effect of Hippophae rhamnoides and Boswellia Extracts in rat's Gastrocnemius muscle. (Magnification: x400) A: Naive group, B: 2% carrageenan injecting group, C: 15 mg/kg of Boswellia extract administering group, D: 30 mg/kg of Boswellia extract administering group, E: 150 mg/kg of Hippophae rhamnoides extract administering group, F: 300 mg/kg of Hippophae rhamnoides extract administering group, G: mixture (15 mg/kg of Boswellia extract and 150 mg/kg of Hippophae rhamnoides extract) administering group.

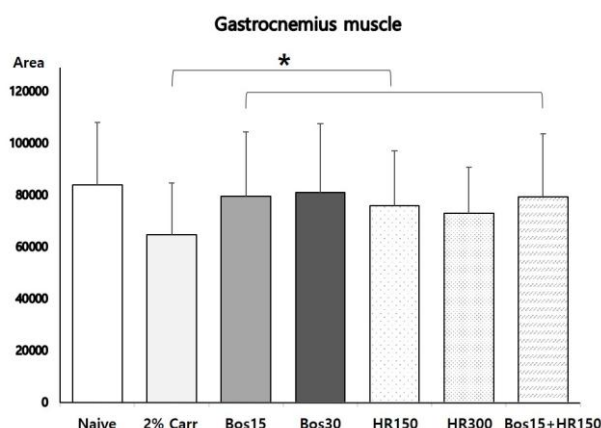


Fig 4. Area of cell in rat's Gastrocnemius muscle. Boswellia and Hippophae rhamnoides Extracts administering groups increased cellular area more than 2% carrageenan injecting group. The cell area tended to increase with the concentration of the administrated extract. however, the cell area of HR150 group was narrow more than the cell area of HR300 group. 2% carr: 2% carrageenan injecting group, Bos15: 15 mg/kg of Boswellia extract administering group, Bos 30: 30 mg/kg of Boswellia extract administering group, HR150: 150 mg/kg of Hippophae rhamnoides extract administering group, HR300: 300 mg/kg of Hippophae rhamnoides extract administering group, Bos15+HR150: mixture of 15 mg/kg of Boswellia extract and 150 mg/kg of Hippophae rhamnoides extract administering group. * $p < 0.05$

DISCUSSION

In the present study, we examined the anti-inflammatory effects of Boswellia and Hippophae extracts, by using the masseter muscle and the gastrocnemius in the rat. The results of the experiment show that the areas of muscle cells in Bos15, Bos30, HR150, HR300, and Bos15+HR150 group was increased in a concentration-dependent manner, compared to that in 2% carrageenan group, after causing inflammatory in both masseter muscle and gastrocnemius in rats and administering Boswellia and Hippophae rhamnoides extracts to them by concentrations. In addition, the space among cells in 2% carrageenan group was also relatively narrow than extracts administering groups (Bos15, Bos30, HR150, HR300, and Bos15+HR150).

In normal muscle tissue, intercellular space is closed, and the cell's form is a polygon. However, in myopathy, the cells turn into a round shape, vary in size, and occur amyotrophy (Goebel et al., 2013). In the present study, the results of observation on both masseter muscle and gastrocnemius with a microscope, after injecting 2% carrageenan to a rat showed the spherical forms of muscle cells and the reduced areas of them. In addition, we observed that the number of rats' scratches on their faces and the contact between their hind legs and ground were decreased, after 2% carrageenan was injected to them, which was not included in the results.

In the present study, we observed that the area of muscle cells in the group in which inflammation in them was caused by injecting 2% carrageenan, after administering *Boswellia* and *Hippophae rhamnoides* extracts to them was wider than that of those in the group in which inflammation in them was caused by only injecting 2% carrageenan. This result might be resulted from less morphological changes in muscle cells, due to anti-inflammatory effects of both *Boswellia* and *Hippophae rhamnoides* extracts. The Boswellic acid, one of main substances in *Boswellia* has been known to have antiphlogistic and analgesic effects and suppress the production of cytokine (Sailer. et al., 1996; Ammon, 2006; Siddiqui, 2011). *Hippophae rhamnoides* is effective in treating inflammation and ulcers and has an inhibitory action on TNF- α , IL-6, NO, etc (Yang. et al., 2002; Yoon MY, 2013; Jiang. et al., 2017). In addition, Choi et al. (2019) noted that nociceptive behavior was prominently alleviated in the experiment group in which both *Hippophae rhamnoides* and *Boswellia* extracts were administered to the faces of white rats, by observing the nociceptive behavior, after causing inflammation in them.

Choi et al. (2019) reported that inflammatory nociceptive behavior was reduced, as the concentration of *Hippophae rhamnoides* and *Boswellia* extracts that were administered to muscle cells was increased, by observing the nociceptive behavior in response to the inflammation in rats. In addition, Kim and Lee (2017) observed that the degree of reduction in the cross-section of muscle fibers and of the agglutination of nucleus in the experiment group was relatively lower than that in the control group, by comparing the experiment group in which *Eucommia ulmoides* Oliver extracts were administered to the left legs of rats with the control group in which normal saline solution was administered to the left legs, after causing disuse muscle atrophy in them. Such a result was also shown when *Hippophae rhamnoides* and *Boswellia* extracts were administered to masseter muscle and gastrocnemius in this study. Although the size of masseter muscle cells was significantly increased, depending on the concentration of both *Boswellia* and *Hippophae rhamnoides* extracts, that of gastrocnemius muscle cells was increased, depending on such concentration, but there was no statistically significant difference in the size of them, as the concentration was varied. In order to verify the effects of single or mixed administration of natural extracts, the areas of masseter muscle cells were more significantly increased in Bos15+Vit150 group, than those in Bos15 and Vit150 group, after administering the mixture of *Boswellia* and *Hippophae rhamnoides* extracts to them. There were, however, no statistically significant differences in the areas of muscle cells among Bos15+Vit150, Bos 30 and Vit 300 group. There were also no statistically significant differences in the areas of gastrocnemius muscle cells between single and mixed administration of extracts.

Muscle fibers differ from region or species (Sciote et al.,

2003), and masseter muscle in rats only consists of type-II fibers, but type-I and II are mixed in limb muscles and the diameters of muscle fibers in limb muscles are shorter than that in masseter muscles (Rowlerson et al., 1983). Bagüés et al. (2016) found that there was no difference in mechanical allodynia between masseter muscle and gastrocnemius, and that the behavioral responses of masseter muscle were changed, depending on the heat hypersensitivity over time, while there was no change in those of gastrocnemius, by observing changes in both masseter muscle and gastrocnemius, according to mechanical, heat and chemical stimuli, after injecting 3%, 6% and 9% carrageenan to them. Mechanical hyperalgesia was induced by carrageenan in masseter muscle and gastrocnemius. The nociceptive behavior of masseter muscle in the carrageenan group was decreased more significantly than that in the control group, depending on chemical hyperalgesia, while the nociceptive behavior of gastrocnemius in the former was increased more significantly than that in the latter. Although the same extracts were administered to both masseter muscle and gastrocnemius in this experiment, the results were different between them, probably because drug effects might be varied according to the region and composition of each muscle. The findings may provide the basic data for the development of therapies using natural substances, based on the position and composition of each muscle.

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