

## Analgesic Effect of Grape Seed Proanthocyanidin Extract in Fibromyalgia Animal Model

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The acidic saline animal model of pain has been suggested to mimic fibromyalgia (FM). Oligomeric proanthocyanidin complexes (OPC) from grape seeds are known to act as an antioxidant. We studied the effects of OPC on the pain threshold in the acidic saline animal model of pain. The left gastrocnemius muscle was injected with 100  $\mu$ l of saline at pH 4.0 under brief isoflurane anesthesia on days 0 and 5. Control rats (n=5) received identical injections of physiological saline (pH 7.2) on the same schedule. Rats (n=10) with acidic saline injection were separated into two study subgroups. After measurement of pre-drug pain thresholds, rats were injected intraperitoneally with either saline or OPC 300 mg/kg. Paw withdrawal thresholds to pressure were again measured 60 min after intraperitoneal injection. Nociceptive thresholds were measured with a Dynamic Plantar Aesthesiometer by applying an increasing pressure to right or left hind paw until the rat withdrew the paw. Compared to baseline (day 0), acid injections produced mechanical hyper-responsiveness on day 7 (pre-drug) in these rats [ $p<0.05$ ]. A potent antihyperalgesic effect was observed when rats were injected intraperitoneally with OPC 300 mg/kg [injected paw,  $p=0.001$ ; contralateral paw,  $p=0.002$ ]. OPC treatment decreased the expression of acid sensing ion channel 3 in the brain motor cortex area on immunohistochemical staining when OPC 300 mg/kg was administered intraperitoneally in the animal model of FM pain [ $p<0.05$ ]. Further research is required to determine the efficacy of OPC treatments in FM pain in humans.

**Key words** : Fibromyalgia, animal model, oligomeric proanthocyanidin complexes (OPC), acid sensing ion channel

### Introduction

Fibromyalgia (FM) is a chronic pain disorder characterized by widespread muscular pain, tenderness, fatigue, unrefreshing sleep, and many other associated symptoms [42]. The precise pathogenesis of FM is not known, but recent research suggests that dysregulated central nervous system (CNS) pain processing appears to play an important role in FM [23]. Spinal dorsal horn neurons undergo central sensitization during tonic input from C-fiber nociceptor afferents, and this phenomenon is, in turn, closely related to a slow temporal summation of activity termed “windup” [9]. This temporal summation or windup is dependent on N-methyl-D-aspartate (NMDA) receptors and substance P synaptic mechanisms within the dorsal horn of the spinal cord [43]. Abnormal sensitization and temporal summation

of second pain (windup) have been described in patients with FM [35]. Oxidative stress and nitric oxide may play an important role in FM pathophysiology, however it is still not clear whether oxidative stress abnormalities documented in FM are the cause or the effect [1,7,24,25].

The acidic saline animal model of pain has been suggested to mimic human chronic pain syndromes such as FM. In this model, repeated intramuscular (IM) injections of acidic saline produce a widespread hyperalgesia that persists without evidence of significant peripheral tissue damage or inflammation, and is believed to be centrally maintained [33]. IM acid injections alter the basal pattern of brain activation in rats [15]. The overwhelming reduction of regional cerebral blood flow (rCBF) in multiple brain structures following acid injections is consistent with reports of decreased rCBF in unstimulated patients with FM [18,21].

Grape seed extract is a natural plant constituent and contains lipids, proteins, carbohydrates, and polyphenols. Proanthocyanidins are the most abundant phenolic com-

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pounds in grape seeds, and are high-molecular-weight polymers comprised of dimmers or trimers of (+)-catechin and (–)-epicatechin [12]. Oligomeric proanthocyanidin complexes (OPC) from grape seeds has more powerful antioxidative activity than other well-known antioxidants, including vitamin C, vitamin E, and gallic acid [2]. OPC has various biological functions such as antibacterial, antiviral, anti-inflammatory, antiallergic, and vasodilatory actions [3].

It has been suggested that OPC is an effective therapy for oxidation-related diseases in animal models, including tumors, atherosclerosis, gastric ulcer, cataract, and diabetic retinopathy [32]. The beneficial effects of OPC in reducing oxidized low-density lipoproteins and postprandial oxidative stress and improving chloasma have been demonstrated in human clinical trials [22,31,45]. However, little is known about the effects of OPC on pain model and FM. To examine whether OPC has therapeutic potentials in the treatment of FM, we examined the effects of OPC on the pain threshold in acidic saline animal model of pain.

### Materials and Methods

All experiments were approved by the Animal Care and Use Committee and were in accordance with the guidelines of the International Association for the Study of Pain policies on the use of laboratory animals.

Fifteen male Sprague-Dawley rats, weighing 250 to 320 g, kept at 12-hour dark-light cycle with free access to standard rat chow and water, were used for the experiments. Animals were brought to the behavioral testing room an hour before the testing to acclimatize them to the testing environment. Behavioral tests were usually done between 10 AM and 3 PM. Left gastrocnemius muscle was injected with 100  $\mu$ l of saline at pH 4.0 under brief isoflurane (2%) anesthesia on day 0 and 5. Control rats (n=5, group 1) received identical injections of physiological saline (pH 7.2) on the same schedule.

Rats (n=10) with acidic saline injection were separated into two study subgroups. After measurement of pre-drug pain thresholds, the rats were injected intraperitoneally with either saline (n=5, group 2) or OPC 300 mg/kg (n=5, group 3) on day 7. OPC, dissolved in saline, was given intraperitoneally to rats. Paw withdrawal thresholds to pressure were again measured 60 min after intraperitoneal injection in the rats. OPC was kindly provided by Hanlim Pharmaceutical Company (Seoul, Korea).

Ipsilateral and contralateral paw withdrawal responses were measured in response to mechanical stimuli at day 0 (baseline), day 7 (pre-drug), and day 7-1 hour (post-drug). The tests were performed by an experimenter blinded to the treatments. Nociceptive thresholds, expressed in grams (g), were measured with a Dynamic Plantar Aesthesiometer (Ugo Basile, Italy) by applying an increasing pressure to right or left hind paw until the rat withdrew the paw. A maximal cut-off value of 50 g was used to prevent tissue damage.

After behavioral testing was completed on day 7, acid injected animals were anesthetized with sodium pentobarbital and perfused with saline solution followed by 4% paraformaldehyde in phosphate buffer. Brains were postfixed for 12h and immersed in 30% buffered sucrose for 5 days. Serial transverse sections of the brain were cut at 30 $\mu$ m in a cryostat. The sections were subjected to an immunostaining assay using substance P (SP) (1:3,000, Immunostar USA), anti-acid sensing ion channel 3 (ASIC3) (1:500, Chemicon USA) and mu opioid receptor (MOR) (1:5,000; rabbit polyclonal Chemicon, Millipore USA) etc. Immunostaining was performed by the LSAB 2 system-HRP kit (Dako, USA). Sections were developed in a substrate solution of 0.05% 3, 3'-diaminobenzidine tetrachloride (Sigma, USA) and several regions of interest (caudate-putamen (CPu), primary and secondary motor cortex (M1 and M2), ventrolateral thalamic nucleus (VL), cingulate cortex, etc.) were examined for changes of pain-related molecules. Immunoreactivity was evaluated by assessing staining intensity visually and grading it as no staining (0), mild (1), moderate (2), or strong (3). The reviewers were blinded. Paired t-tests compared mean differences between two groups.

The threshold was tested five times for each time point and the mean was used as the final data. For determining the effect of the drugs on pain threshold, the pressure to paw withdrawal was expressed as g. One-way analysis of variance followed by posthoc analysis for multiple comparisons was used to evaluate the differences between groups for each time point. t-test was used to compare the data of day 7-1 hour between group 2 and group 3. Differences were considered statistically significant at  $p < 0.05$ .

### Results and Discussion

Compared to baseline (day 0), acid injections produced mechanical hyper-responsiveness on day 7 (pre-drug) in

these rats [ $p < 0.05$ ] (Fig. 1 and 2). A potent antihyperalgesic effect was observed when rats were injected intraperitoneally with OPC 300 mg/kg [injected paw,  $p = 0.001$ ] (Fig. 1) and [contralateral paw,  $p = 0.002$ ] (Fig. 2). This finding was observed at 60 min of the treatment (Fig. 1 and 2).

Compared to intraperitoneal sterile saline group, OPC group showed that a decrease of expression of ASIC3 in brain M1 and M2 area was observed on immunohistochemical

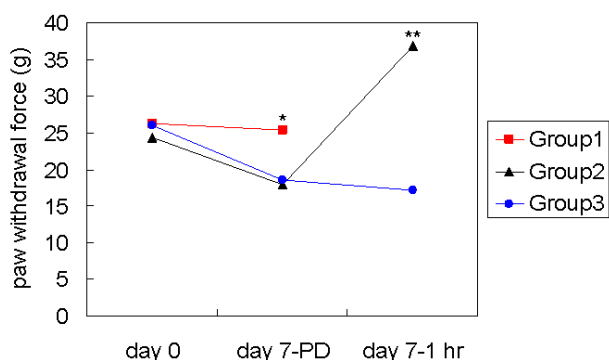


Fig. 1. Effect of OPC on the pain threshold in the acid injected paw of the rats (n=15). Compared to baseline (day 0), acid injections produced mechanical hyper-responsiveness on day 7-PD ( $p < 0.05$ ). A potent antihyperalgesic effect was observed when OPC was injected intraperitoneally [day 7-1 hr] ( $p = 0.001$ ). PD, pre-drug; gp, group; hr, hour; Group1, pH 7.2 saline IM only; Group2, pH 4 saline IM + intraperitoneal OPC 300 mg/kg; Group3, pH 4 saline IM + intraperitoneal sterile saline (vehicle control). \*  $p < 0.05$ , \*\*  $p = 0.001$

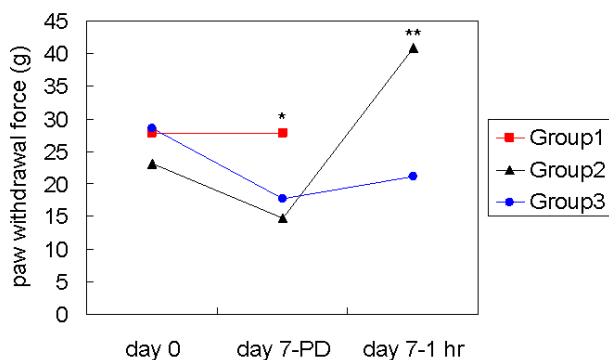


Fig. 2. Effect of OPC on the pain threshold in the contralateral paw of the rats (n=15). Compared to baseline (day 0), acid injections produced mechanical hyper-responsiveness on day 7-PD ( $p < 0.05$ ). A potent antihyperalgesic effect was observed when OPC was injected intraperitoneally [day 7-1 hr] ( $p = 0.002$ ). PD, pre-drug; gp, group; hr, hour; Group1, pH 7.2 saline IM only; Group2, pH 4 saline IM + intraperitoneal OPC 300 mg/kg; Group3, pH 4 saline IM + intraperitoneal sterile saline (vehicle control). \*  $p < 0.05$ , \*\*  $p = 0.002$

staining when OPC 300 mg/kg was administered intraperitoneally in animal model of FM pain [ $p < 0.05$ ] (Fig. 3). There was no difference in the expression of ASIC3 on another areas of interest (CPu, VL, cingulate cortex, etc.). There was also no difference in the expression of SP and MOR on regions of interest (M1, M2, CPu, VL, cingulate cortex, etc.).

In this study, unilateral IM injections of acidic saline produced bilateral mechanical hyper-responsiveness in Sprague-Dawley rats. In this model, a potent antihyperalgesic effect was observed when rats were injected intraperitoneally with OPC 300 mg/kg. A increase of pain threshold was observed not only in the injected side paws but also in the non-injected side paws of rats with hyperalgesia in the current study. OPC treatment decreased the expression of ASIC3 in brain M1 and M2 area on immunohistochemical staining when 300 mg/kg was administered intraperitoneally in animal model of FM pain.

There has been no established experimental animal model for FM, but the acidic saline animal model of pain has been suggested to mimic FM [33]. Sluka et al showed that the activation of ASIC3 on muscle afferents was required for development of mechanical hyperalgesia and central sensitization that normally occurred in response to repeated IM acid [34]. Russell et al demonstrated that SP was elevated in the cerebrospinal fluid of patients with FM [28]. This finding was replicated in other neuropeptide studies [29]. Abnormal sensitization and temporal summation of second pain have been described in patients with FM [35]. This temporal summation is dependent on SP synaptic mechanisms within the dorsal horn of the spinal cord [43]. Therefore, we chose SP, ASIC3, and MOR [29] for the examination of pain-related molecules.

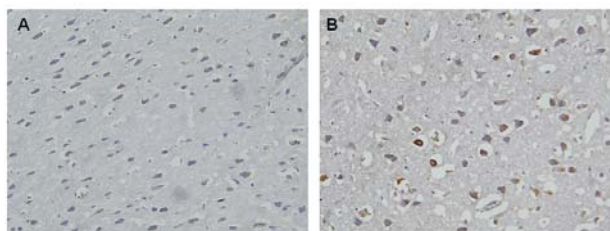


Fig. 3. Immunohistochemical staining showing the expression of ASIC3. Compared to sterile saline treatment (B), OPC treatment (A) decreased the expression of ASIC3 in brain M1 and M2 area when 300 mg/kg was administered intraperitoneally in animal model of FM pain ( $p < 0.05$ ). ( $\times 400$ )

Acid-sensing ion channels (ASIC) are proton-gated cation channels highly expressed in peripheral sensory and central neurons [17,38]. These channels are thought to serve important roles in a number of different physiological and pathological processes. In sensory neurons, ASIC are implicated in nociception [6,37], mechanosensation [26,27]. In central neurons, ASIC are involved in synaptic plasticity, learning-memory, and fear conditioning [39-41]. In addition, these channels play central roles in neuronal hyperexcitability and hyperalgesia [19,34]. While ASIC1a, 2a, 2b, and 4 are widely expressed in both central and peripheral sensory neurons, ASIC1b and 3 are found primarily in sensory neurons [17,38].

Hyperalgesia is considered as integration of changes occurring in multiple regions of CNS, a massive integrated neural network [5,30]. Thus, CNS should be investigated more widely. Although electrophysiological techniques are useful for investigation of the pain modulation system, it is still difficult to surveil molecules functioning in the system. From these viewpoints, Kim *et al.* reported that compared to controls, acid injected rats demonstrated increased expression of several pain-associated molecules in specific structures [16]. Unilateral IM injections of acidic saline altered the basal pattern of activation in multiple CNS structures [15]. These data support the validity of the acidic saline assay as a model of FM, and provide a foundation for future analysis of the contribution of specific brain structures and cellular mechanisms to the development and maintenance of central pain syndromes.

In a recent study, statistical comparison of the patient and control groups receiving similar stimulus pressures revealed 13 regions of greater activation in the patient group [13]. In a comparison of the effects of similar stimulus pressures, patients showed significant activations that were significantly different from the activation in the healthy controls in the primary somatosensory cortex, inferior parietal lobule, insula, cingulate cortex, secondary somatosensory cortex, superior temporal gyrus, and cerebellum. Therefore, we chose several regions of interest (cingulate cortex, putamen, motor cortex etc.) for the examination of pain-related molecules.

The motor cortex (M1 and M2) is involved in pain modulation [44]. The distribution of SP in mid- and fore-brain areas of the rat was studied using a radioimmunocytochemical method [20]. An extensive distribution of SP was seen in frontal, cingulate, retrosplenial, and entorhinal cortices.

There is no report of ASIC3 in M1 and M2 of human and animal till now.

Pregabalin has been shown to be efficacious in other pain conditions including postoperative pain and FM. Specifically, in people with FM, pregabalin (450 mg/d) reduced pain, improved sleep, and increased quality of life [8]. More recently, it is reported that pregabalin reduced muscle and cutaneous hyperalgesia in acid saline model of rats [46]. There is no study of antioxidant treatment in acid saline model till now. We chose 300 mg/kg of OPC as intraperitoneal injection dose because there were no differences at 50 and 100 mg/kg in preliminary study. There was no study of using over 300 mg/kg of OPC in rats as we reviewed all available literatures about this.

Reactive oxygen species (ROS) such as the superoxide anion ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), and the hydroxyl radical, are continuously generated during normal cell metabolism. ROS can act as a physiological defense system against microbial infection and are involved in maintaining normal cellular functions, including proliferation, apoptosis, and intracellular signal transduction [11]. However, excessive amounts of ROS can damage lipids, proteins, DNA, and the extracellular matrix [10]. Therefore, ROS must be scavenged by antioxidants. Enzymatic antioxidants include superoxide dismutase, glutathione peroxidase, catalase, and thioredoxin reductase. Nonenzymatic antioxidants include glutathione, vitamin A, vitamin C, and vitamin E [36]. Oxidative stress refers to the cellular state in which the production of ROS is elevated and/or the levels of antioxidants reduced. Oxidative stress is considered to be involved in the pathogenesis of atherosclerosis, cancer, neurodegenerative disorders, diabetes, ageing, and autoimmune rheumatological diseases [36]. The polyphenols in OPC have shown beneficial antioxidant effects on oxidative-stress-associated diseases. It has been suggested that oxidative stress plays an important role in the pathobiology of FM [1,7,24,25].

Nitric oxide (NO) is an important cell-cell signaling molecule and a free radical [7]. NO plays an important role in many biochemical processes such as regulation of blood vessel dilatation and immune response, and functions as a neurotransmitter [7]. NO can interact with superoxide to form peroxynitrite, a highly toxic compound that can nitrosylate proteins and alter their function [4]. Temporal summation of second pain or wind-up is mediated via NMDA receptors and may be involved the production of NO [14]. Wind-up depends on input from unmyelinated C fiber afferents that

can activate central NMDA receptor systems associated with enhancement of pain. In this FM model, repeated IM injections of acidic saline produce a widespread hyperalgesia which might be centrally maintained [33]. As central sensitization play a pivotal role in clinical pain for FM and NMDA receptors are involved in NO production [7], we suggest that antioxidant effect of OPC might be helpful for relieving a widespread hyperalgesia.

The mechanism of this stress-induced modulations of ASIC3 expression remains unknown. In our study, the role of OPC about decreased ASIC3 expression in M1 and M2 of hyperalgesic rats also remains to be determined. It would also be interesting to know, whether the alterations in ASIC3 mRNA expression are long-lasting or transient, or whether repeated stress exposure can lead to progressive changes in the expression of this gene in the future study.

We provide here, first evidence that IM acid injections altered the expression pattern of ASIC3 in M1 and M2 of rats. The change in expression of ASIC3 that was observed in specific brain structure following acid injections looks like associated with reports of changed pain-associated molecules in patients with FM [29]. Therefore, interfering with ASIC3 might be of benefit in treatment or prevention of chronic hyperalgesia. These data support the validity of acidic saline treatment as a model of FM, and provide a foundation for future analyses of specific brain regions and molecules that contribute to this syndrome. In conclusion, we have demonstrated that a potentiation in the anti-hyperalgesic effect is observed when OPC were administered in animal model of FM pain. This should encourage further researches evaluating the potential role of oxidative stress in the pathophysiology of FM and the efficacy of antioxidant treatments in double blind and placebo controlled trials. These future researches will enhance our understanding of the complex pathophysiology of this disorder.

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초록 : 섬유근통 동물 모델에서 포도씨 추출 proanthocyanidin의 진통 효과

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산성 식염수 쥐모델은 사람의 섬유근통에 근접한 모델로 제시되고 있다. 포도씨에서 얻은 oligomeric proanthocyanidin complexes (OPC)는 항산화제로 알려져 있다. 저자들은 산성 식염수 모델에서 통증 역치에 대한 OPC의 효과를 연구했다. 좌측 장딴지 근육에 pH 4.0의 산성 식염수 100  $\mu$ l를 0일과 5일에 주사했다. 대조군은 pH 7.2의 생리 식염수를 같은 스케줄로 주사했다. 산성 식염수 그룹 10마리를 다시 두 그룹으로 나누어 한 그룹은 멸균 식염수, 다른 한 그룹은 OPC 300 mg/kg를 복강 내 주사했다. 복강 내 주사 한시간 후 다시 통각에 대한 역치를 조사했다. 0일에 비해 7일에서 산성 식염수 모델은 기계적 과통각을 나타냈다( $p < 0.05$ ). OPC 300 mg/kg를 복강내 주사한 그룹에서 강력한 항통각 효과를 나타냈다(주사측 발바닥,  $p = 0.001$ ; 반대측 발바닥,  $p = 0.002$ ). 면역조직화학 염색상 복강내 식염수를 처치한 대조군에 비해 OPC 처치군에서 대뇌의 M1 및 M2 영역에서 산-감지 이온 통로3의 발현이 감소되었다( $p < 0.05$ ). 사람의 섬유근통에서 OPC 치료의 효과를 보기 위한 연구가 향후 필요할 것으로 생각된다.