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Ameliorating Effects of *Jixueteng* in a Mouse Model of *Porphyromonas gingivalis*-Induced Periodontitis: Analysis Based on Gingival Microcirculatory System

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Jixueteng, the dried stem of *Spatholobus suberectus* Dunn (Leguminosae), is a traditional Chinese herbal medicine that promotes blood circulation and can be used to treat blood stasis. In this study, we aimed to investigate the potential of *Jixueteng* as a preventive and therapeutic drug for periodontitis. We investigated the inhibitory effects of *Jixueteng* on *Porphyromonas gingivalis* (*P. gingivalis*)-induced gingival circulatory disturbances in mice. Seventy-two male C57BL/6N mice (4-week-old) were randomly divided into the following four groups of 12 mice each. Group 1 served as the *P. gingivalis* noninfected control (control group). Group 2 was administered *Jixueteng* extract in drinking water to *P. gingivalis* noninfected control mice. Group 3 was infected orally with *P. gingivalis*; and group 4 was administered *Jixueteng* extract in drinking water and then infected with *P. gingivalis*. To evaluate the effect of *Jixueteng* on gingival microcirculation systems, we examined gingival blood flow (GBF) in oral microcirculation *in vivo* in a mouse model of periodontitis. Gingival reactive hyperemia (GRH) was determined using laser Doppler flowmetry. GRH was elicited by the release of occlusive gingival compression by the laser Doppler probe (diameter 1.0 mm) for 1 min. GRH was estimated by basal blood flow, maximum response (Peak), the time taken for the maximum response to fall to one half ($T_{1/2}$) and increased total amount of blood flow (Mass). Furthermore, to determine the effect of an oral application of *P. gingivalis* and/or *Jixueteng* on GBF and gingival microvessel ultrastructure, morphological analysis of gingival microvessels was performed by using a vascular resin cast model. Administration of *Jixueteng* to *P. gingivalis*-infected animals significantly reduced GRH, especially $T_{1/2}$ and Mass, compared to that in *P. gingivalis*-infected animals. Alternatively, in the morphological analysis, reduction of the gingival capillary network which resulted from *P. gingivalis*-infection was improved by *Jixueteng* administration. Since *Jixueteng* ameliorates *P. gingivalis* infection-induced gingival circulatory disturbance, it may be useful in the treatment of *P. gingivalis*-induced periodontitis.

Keywords: *Jixueteng*, Herbal medicine, Gingival reactive hyperemia, Gingival microcirculation, Laser Doppler blood flowmetry.

Periodontitis is a major disease in the dental field. Dental plaques comprising specific oral indigenous bacteria, such as *Mutans streptococci* and *Porphyromonas gingivalis* (*P. gingivalis*), are the direct causes of periodontitis [1, 2]. Chronic (adult) periodontitis is an infection induced in periodontal tissues by the proliferation of gram-negative obligate anaerobic bacteria from among the bacterial flora of the gingival sulcus [1, 2]. Prevalence increases with aging, and most people in their 40s or above have localized or pervasive periodontitis [3, 4].

P. gingivalis is typically detected at a high frequency in patients with periodontitis. It contains an endotoxin possessing potent proteolytic and bone resorption-inducing activities that causes gingival microcirculatory disturbance, suggesting that it causes chronic periodontitis. It has recently been clarified that aggravation of periodontal disease is not only associated with local inflammation and tooth loss due to alveolar bone resorption, but also with systemic diseases accompanied by circulatory failure, such as endocarditis, arteriosclerosis, diabetes, obesity, osteoporosis, and aspiration pneumonia [5, 6].

The prevention and treatment of periodontitis largely depend on mechanical cleaning using a toothbrush and surgical treatment. For the prevention and treatment of oral diseases mainly caused by dental plaques, such as dental caries and periodontitis, growth inhibition and removal of dental plaques and oral bacteria are important. However, complete removal of plaques is difficult even by appropriate brushing and requires advanced techniques. In addition, the recent rapid spread of dental implants has led to peri-implantitis accompanied by inflammatory symptoms around implants, similar to symptoms of chronic periodontitis [7, 8]. Therefore, improved prevention and treatment methods for periodontal diseases are needed.

Since changes in the number of *P. gingivalis* in periodontal pockets are consistent with the pathology of chronic periodontitis, substances with a direct antimicrobial action or inhibitory action against inflammatory circulatory failure of gingival microcirculation may be effective in preventing and inhibiting the progression of chronic periodontitis.

Jixueteng is an herbal medicine prepared by drying the stems of *Spatholobus suberectus* Dunn belonging to the Leguminosae family. It shows pharmacological actions such as improvement of circulation, analgesia, and normalization of red and white blood cell count. Recently, we discovered that *Jixueteng* has potent active radical

scavenging [9], antimicrobial [10], and alveolar bone resorption-inhibitory [11] activities. In this study, the effect of *Jixueteng* on periodontitis was clarified by analyzing gingival microcirculation regulatory dynamics using a mouse model of *P. gingivalis*-infected periodontitis. In addition, the possibility of the clinical application of this herbal medicine in the dental field was investigated.

Measurement of GBF: No remarkable alteration was observed in all GBF parameters (Peak, Mass, and $T_{1/2}$) before *P. gingivalis* infection among the four groups (Figure 1). At 53 days after *P. gingivalis* infection (10 weeks old), significant increases were observed in $T_{1/2}$ and Mass in the mouse model for periodontal disease (*P. gingivalis* infection; *P. g* in Figure 1C, D). Subsequently, Mass and $T_{1/2}$ increased with time in these mice, whereas no change was noted in any of the parameters in (*P. gingivalis* infection + *Jixueteng* administration; *P. g* + *Jix* in Figure 1C, D), and the values were close to those in the control group. These results indicate that the microvessels of the gingival tissue tends to dilate after *P. gingivalis* infections.

Preparation of vascular cast: In the mouse model of experimental periodontitis, density decreased with time owing to destruction of capillary blood vessel network in periodontal tissue in the *P. gingivalis*-infected group (Figure 2C) compared with that in the non-*P. gingivalis*-infected group (Figure 2A) and in the non-*P. gingivalis*-infected + *Jixueteng* treatment group (Figure 2B). In contrast, in the *P. gingivalis*-infected + *Jixueteng* treatment group (Figure 2D), density was retained at a level similar to that in the non-*P. gingivalis*-infected group, and there was no reduction in density (Figure 2D).

In this study, we aimed to investigate the potential of *Jixueteng* as a preventive and therapeutic drug for periodontitis and found that *Jixueteng* exerted ameliorating effects on *P. gingivalis* infection-induced gingival circulatory disturbance.

Herbal medicines are used in modern medical care to improve symptoms of intractable diseases such as cancer and lifestyle-related diseases such as hypertension and diabetes. The concept of oral medicine originated recently. Various oral diseases are systemically evaluated and noninvasive approaches, including drug therapy, are employed to treat symptoms such as pain, dry mouth, bad breath, and dysgeusia. The use of herbal medicines widely broadens the possibility, range, and means of treatment, and marked improvement of symptoms by their application has been frequently reported.

Authentic *Jixueteng* is listed in the Chinese Pharmacopoeia. However, more than 30 plant species have been used under the general name ‘‘*Jixueteng*’’ in traditional Chinese and folk medicine [12, 13]. *Jixueteng* is a traditional Chinese medicine that improves, increases, and tones blood circulation [14]. It is also used to treat irregular menstruation, blood deficiencies, and rheumatalgia [15, 16]. *Jixueteng* comprises various components, predominantly polyphenols [17-19]. Previous chemical and pharmacological investigations have indicated that flavonoids are the main ingredients of *S. supercuts* [20, 21].

Although *Jixueteng* is not frequently used in Japan, we focused on the potent radical scavenging ability of *Jixueteng.* The inhibitory effect of *Jixueteng* on gingival microcirculation systems in periodontal disease caused by oral bacteria was investigated (Figure 1). Ingestion of its decoction not only improves blood flow through blood replenishment and strengthening of immunity but also recovers the autonomous nervous system and visceral function and

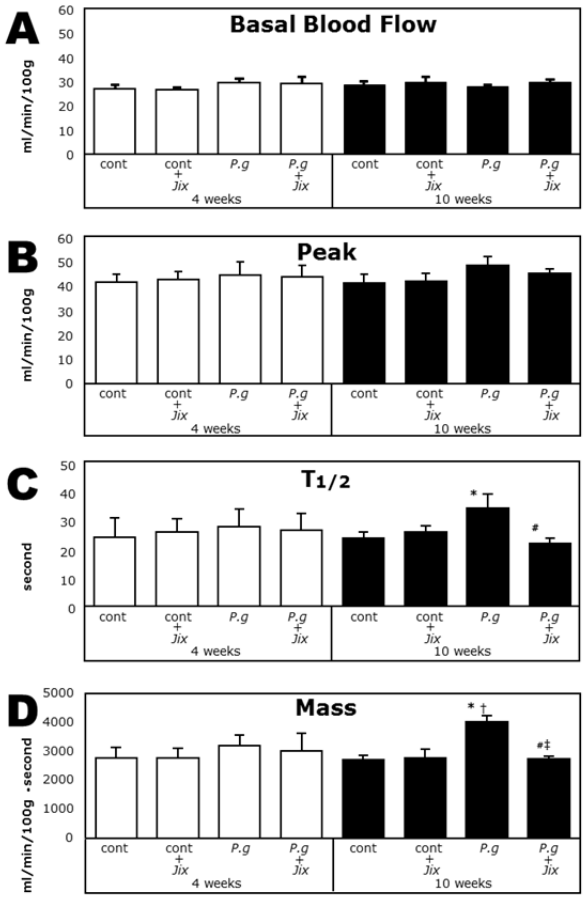

Figure 1: Effect of *P. gingivalis* infection and/or *Jixueteng* administration on mouse GBF and gingival reactive hyperemia. No remarkable alteration was observed in all GBF parameters (Basal blood flow, Peak, Mass and $T_{1/2}$) before *P. gingivalis* infection among the four groups (control, control + *Jixueteng*, *P.g*, *P.g* + *Jixueteng* on 4weeks aninal models). Significant increases were observed in $T_{1/2}$ and Mass in the mice model of periodontal disease after *P. gingivalis* infection (*P.g*) at 10 weeks (C, D). A remarkable change was noted in all the parameters in between the periodontal disease model mice (*P.g*) and *Jixueteng*-treated mice (*P.g* + *Jix*), and the values were close to those in the control group (C, D). Data are presented as mean ± SEM (n=4-6). **P*<0.05 *vs*. 10 weeks control, #*P*<0.05 *vs*. 10 weeks *P. gingivalis*, †*P*<0.05 *vs*. 4 weeks control, ‡*P*<0.05 *vs*. 10 weeks *P. gingivalis* + *Jixueteng*.

improves ataxia of the endocrine system[9]. It has recently been shown that *Jixueteng* has antioxidant and antimicrobial activity, such as antiviral action on enterovirus and inhibitory action on protease activity of type 1 human immunodeficiency virus, in addition to hematopoiesis through activation of granulocytes and megakaryocytes [22-24]. In addition, the potential of *Jixueteng* as a cosmetic material has been suggested, including tyrosinase inhibitory activity, which can inhibit melanin synthesis by human epithelial melanocytes [25].

The test bacteria *P. gingivalis,* which is the direct cause of periodontal disease, colonizes under anaerobic conditions developed due to plaque formation, which is a direct consequence of poor oral hygiene. It plays an important role in the development and progression of periodontitis owing to its potent proteolytic activity and lipopolysaccharide content, with strong alveolar bone resorption ability.

P. gingivalis and *F. nucleatum* co-agglutinate through galactose residues and lectin-like proteins. *Jixueteng* extract may interfere with symbiotic relationships, such as bacterial aggregation, which

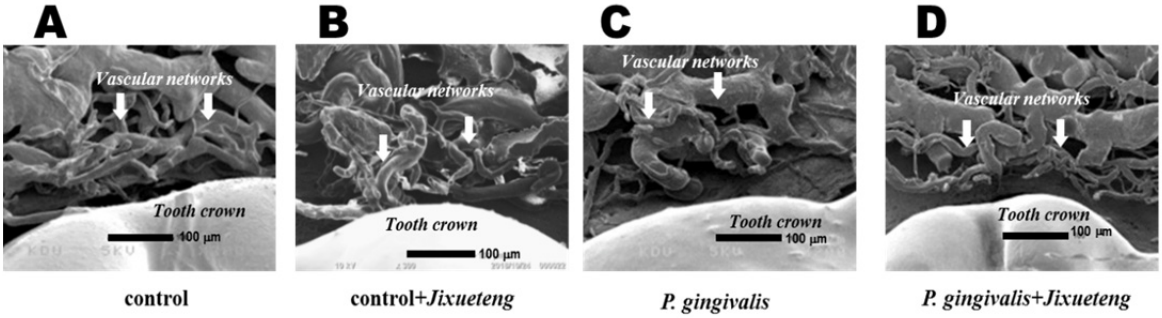

Figure 2: Scanning electron microscopy. Compared to the normal control group (A). Gingival vascular networks in control + *Jixueteng* treatment rarely changes with the normal group (B), morphological degeneration of vessels in vascular networks and abnormality of the vascular lumen caused by *P. gingivalis* infection were observed (C). However, improvement in the degeneration of these vascular networks and prolongation of the vascular plexus were observed by administration of *Jixueteng* (D).

may be effective in inhibiting dental plaque formation and treating periodontal disease [9-11]. We previously reported the use of animals orally infected with *P. gingivalis* as a chronic inflammation model. We suggested that *P. gingivalis*-induced alveolar bone loss could occur in periodontitis and in "hypertension and stroke" animal models, such as SHRSP [26]. Furthermore, the reactivity of blood vessels in the oral cavity was modulated by *P. gingivalis* infection in this experiment. As described above, *P. gingivalis* is found in the lesions of atherosclerosis and can also penetrate the vascular endothelium [27]. In addition, vascular contraction is affected by reactive oxygen species [28, 29]. In this study, we employed a *P. gingivalis*-infected mouse periodontitis model to evaluate the effect of *Jixueteng* on periodontitis by analyzing gingival microcirculation regulatory dynamics. $T_{1/2}$ and Mass in *P. gingivalis*-infected mice increased compared to the controls. This increase was prevented by pretreatment with *Jixueteng* (Figure 1). GBF might depend on the modulation of gingival vascular endothelium function in the *P. gingivalis* infection models. As a result of GBF measurements, increases in post ischemic-reperfusion blood flow (RH) represent an efficient vasodilatory response to ischemic tissues following the temporary occlusion of blood vessels, i.e., a compensatory circulation reaction. Humoral, myogenic, and urogenic factors have been suggested to play roles in vasodilatory regulation in RH [30-32].

We previously demonstrated that nitric oxide (NO) was a mediator of RH in gingiva of several kinds of animals [33-35]. Therefore, NO secreted by the nitrergic nerve as a paracrine agent is considered to play a role in rat gingival RH. Flow-mediated dilation (FMD) involving vascular avascularization and release is considered suitable for non-invasive assessments of vascular endothelial function and stiffness via NO and other vasodilators. Furthermore, this method has already been applied to humans in clinical settings for the early detection and treatment of lifestyle-related diseases such as hypertension, arterial sclerosis, and diabetes.

Although the relaxation response was increased in the GRH of *P. gingivalis*-infected model, the reaction, inhibited by *Jixueteng* (Figure 1), was reversible. This also confirmed that superoxide anion ($O_2^{\cdot-}$) would be involved. This may be caused by a relative reduction of $O_2^{\cdot-}$ involved in contractions via reactions between NO and $O_2^{\cdot-}$. One cause may be a large amount of non-physiological NO derived from inducible nitric oxide synthase as it is induced in macrophages stimulated by the lipopolysaccharides of *P. gingivalis*. As can be inferred from the results, blood vessels relax when *P. gingivalis* infection is induced, which may present as restored reactivity of the blood vessels. In previous experiments, *P. gingivalis* infection induced in SHRSP decreased blood pressure, consistent with our results [26]. The results of Figure 1 in the present study are similar to those previously reported [34]. The vasodilatation due to the *P. gingivalis* infection is improved by *Jixueteng* administration (Figure 1C). In addition, the vasodilatory trend by NO derived from *P. gingivalis* infection is observed with the vascular cast specimen of Figure 2A, B. Furthermore, *Jixueteng* prevented the reduction in the number of the gingival microcirculation networks due to *P. gingivalis* infection (Figure 2B). The antioxidant activity, circulatory improvement, antibacterial effects of *Jixueteng* likely contribute to these effects (Figure 1C and 2C). However, if this phenomenon is caused by the balance between physiologic or non-physiologic (*P. gingivalis* infection-induced) NO and $O_2^{\cdot-}$, inflammatory vascular dysfunction caused by ROS may be simultaneously progressing, while suggesting an apparent recovery.

Moreover, morphological analysis using the vascular resin cast model gingival microvessel structure was damaged by *P. gingivalis* infection, but this damage was prevented by *Jixueteng* administration (Figure 2). Since *Jixueteng* improves blood circulation and has hematopoietic activity, systemic treatment with *Jixueteng* may lead to functional improvement, including improvement of circulatory disorders in periodontitis lesions. *Jixueteng* preparations applicable for local treatment of periodontitis lesions, such as ointments and oral rinses, have been developed. Utilization of *Jixueteng* in dental medicines may allow simultaneous oral and systemic hygiene management.

Experimental

Experimental design: Seventy-two male C57BL/6N mice (4-week-old; each weighing 20 g) were obtained from a commercial farm (Nihon SLC, Shizuoka, Japan) and housed in cages throughout the experimental period when periodontitis was induced; the animals were thus successfully kept in isolation. They were fed a standardized diet of hard briquettes and water and maintained under a 12-h light/dark cycle (lights on at 8:00 am and off at 8:00 pm) at a temperature of 22°C ± 3°C and a relative humidity of 50%. As shown in Figure 3, the mice were given sulfamethoxazole (1 mg/mL; Sigma-Aldrich, St. Louis, MO) and trimethoprim (200 mg/mL; Sigma-Aldrich) in their drinking water for 4 days to reduce original oral flora, followed by a 3-day antibiotic-free period before inducing *P. gingivalis* infection.

The mice were randomly divided into four groups of 12 mice each (with six mice each used for GBF measurement and morphological analysis). Mice in the same group were housed together. Group 1 served as the *P. gingivalis* non-infected native control (control).

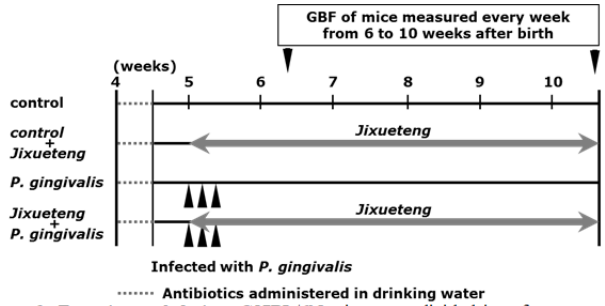

Figure 3: Experimental design. C57BL/6N mice were divided into four groups (6 mice/group). Group 1, control (non-challenged with *Porphyromonas gingivalis*); Group 2 was administered *Jixueteng* extract in drinking water to *P. gingivalis* noninfected control mice. Group 3 was infected orally with *P. gingivalis*; and group 4 was administered *Jixueteng* extract in drinking water and then infected with *P. gingivalis*.

Group 2 was administered *Jixueteng* extract in drinking water to *P. gingivalis* noninfected control mice (control + *Jixueteng*). Group 3 was infected orally with *P. gingivalis* (control + *P. gingivalis*); and group 4 was administered *Jixueteng* extract in drinking water and then infected with *P. gingivalis* (*Jixueteng* + *P. gingivalis*). The mice were infected orally with *P. gingivalis*, which was suspended in 5% carboxymethylcellulose (CMC; Sigma-Aldrich). Each mouse received 0.1 mL (1.0×10^{10} cells/mL) of the suspension by oral gavage (three times) at 48-h intervals. After bacterial infection, six mice were selected from each group and killed to examine the change in gingival microcirculation systems by vascular resin cast model at six weeks after *P. gingivalis* infection (end of the experiment) [11]. Every effort was made to minimize animal suffering and reduce the number of animals used in this study. The experimental procedures of this study were reviewed and approved by the Committee of Ethics on Animal Experiments of Kanagawa Dental University. All experiments followed the Guidelines for Animal Experimentation of Kanagawa Dental University.

Preparation of Jixueteng extract: *Jixueteng* is the generic name of a species within the legume family. The dried vines of *S. suberectus* Dunn (Tochimoto tenkaido Co., Ltd., Osaka, Japan) were used in this study. The sample (200 g) was extracted by boiling in 1 L of distilled water for 3 h, followed by filtration to remove debris just before experimental procedures. *Jixueteng* extract was diluted with phosphate buffered saline (PBS; pH 7.4) for use in the experiments. An 8% extract was prepared and evaluated for its efficacy in experimental periodontitis and antibacterial activity against *P. gingivalis* [9].

Gingival blood flow: All mice were anesthetized with sodium pentobarbital (45 mg/kg, *i.p.*) and were subsequently given small maintenance doses, as necessary. After determination of body weights, GBF was measured at the palatal gingiva by a laser Doppler flowmeter (TBF-LN1, Unique Medical Co., Ltd., Tokyo, Japan) with a laser Doppler probe (diameter 1.0 mm). GRH was elicited by the release of occlusive gingival compression by the laser Doppler probe for 1 min. As shown in Figure 4, GRH was estimated by basal blood flow, maximum response (Peak), the time taken for the maximum response to fall to one half ($T_{1/2}$) and increased total amount of blood flow (Mass)[33-35]. The output signals from the flowmeter were recorded on a computer hard disc through an A/D converter and displayed simultaneously on the monitor. The recorded GBF data were analyzed using a data analysis software (Chart v 4.2 AD Instruments, Inc., Colorado Springs, CO, USA). These GBF measurements were performed on day six after the purchase of mice for all four groups before *P. gingivalis* infection. In addition, other GBF measurements were performed once a week in the mice of all groups except Group 1,

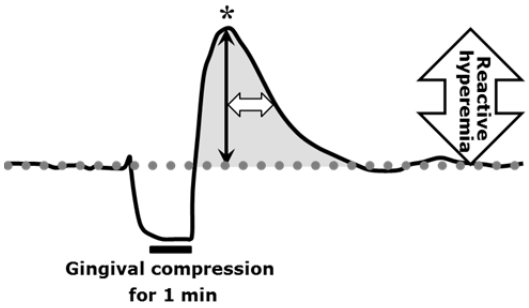

Figure 4: Typical trace of GBF during reactive hyperemia. Reactive hyperemia was elicited by the release of occlusive gingival compression by the laser Doppler probe for 1 min. Typical trace of GBF during reactive hyperemia illustrating the basal blood flow (gray dotted line), maximum response (* Peak), time taken for the maximum response to fall to one half (open arrow: $T_{1/2}$), and increased total amount of blood flow (shaded area; Mass). GBF: gingival blood flow.

native control when they were 6–10 weeks old for analysis of the effects of *Jixueteng* administration, and/or *P. gingivalis* oral infection. The animals were returned to the same cage after GBF measurements. The trace presented in Figure 4 is the typical result.

Vascular resin cast model: All mice were anesthetized with sodium pentobarbital (45 mg/kg, *i.p.*) and killed when they reached 10 weeks. The common aortic arch of each group was cannulated, and Ringer's solution containing 0.2% heparin was perfused until the right atrial veins were cleared of blood. After perfusion, 2% glutaraldehyde phosphate buffer solution (pH 7.4) was injected into the aortic arch for tissue fixation. Following fixation, synthetic resin (Mercox®, Dai Nippon Ink, Tokyo, Japan) was injected manually into the general vascular systems. The soft tissue was digested by incubating the blocks at 40°C for approximately two weeks in PBS (pH 8.4) containing 20% proteinase (Prozyme 6®, Amano, Nagoya, Japan). All the specimens were then washed thoroughly with 40°C tap water and freeze-dried. After being ion-coated with platinum–palladium, the specimens were examined using a scanning electron microscope (SEM) (JSM6301F, JEOL, Akishima, Japan) [36]. All SEM images presented are typical results.

Statistical analysis: Analysis of variance (ANOVA) and multiple comparison tests using Tukey's method were applied to determine the differences between groups. For comparison of only one pair, Student's paired *t*-test and ANOVA were used. Data are expressed as the mean ± SEM. A p value less than 0.05 was considered statistically significant. Data were analyzed with Microsoft Excel 2013.

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Conflict of Interest - No potential conflicts of interests were disclosed.

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