Anti-inflammatory and Antioxidant Effect of Poly-gallic Acid (PGAL) in an In Vitro Model of Synovitis Induced by Monosodium Urate Crystals

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Abstract—Gout is a chronic and degenerative disease that affects the joints and soft tissues because of the crystalline deposit of monosodium urate. The interaction between monosodium urate crystals (MSU) and synoviocytes generates oxidative and inflammatory states. These physiological characteristics have promoted the study of poly-gallic acid (PGAL), a poly-oxidized form of gallic acid reported to be effective in in vitro models of inflammation. The effect of PGAL in an in vitro model of oxidation and synovial inflammation induced by MSU was evaluated after 24 h of stimulation through the morphological changes, the determination of oxidative stress (OS), IL-1β, and the phagocytosis of the MSU. A 20% reduction in synovial viability and the generation of vesicles were observed when they were exposed to MSU. When PGAL was used at 100 and 200 µg/ml, cell death was reduced by 30% and 17%, respectively. PGAL both doses reduce the vesicles generated by MSU. OS generation in synoviocytes exposed to 100 µg/ml and 200 µg/ml PGAL decreased by 1.28 and 1.46 arbitrary fluorescence units (AFU), respectively, compared to the OS in synoviocytes exposed to MSU (1.9 AFU). PGAL at 200 µg/ml inhibited IL-1β by 100%, while PGAL at 100 µg/ml inhibited IL-1β by 66%. The intracellular MSU decreased in synoviocytes stimulated with 100 µg/ml PGAL. The PGAL has a cytoprotective effect against damage caused by MSU in synoviocytes and can counteract the oxidative and inflammatory response induced by the crystals probably because it exerts actions at the membrane level that prevent phagocytosis of the crystals.

KEY WORDS: Poly-gallic acid; Monosodium urate crystals; Phagocytosis; Oxidative stress

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INTRODUCTION

Uric acid is the final product of purine metabolism; it is soluble in the cytosol and in plasma at concentrations lower than 6.8 mg/dl. However, in the extracellular environment and in tissues, mainly in the joints, it can crystallize when it exceeds its saturation limit, result of the establishment of hyperuricemia in the body [1, 2]. Hyperuricemia plays an important role in the pathogenesis of certain processes, such as hypertension, insulin resistance, type II diabetes, and cardiovascular and cerebrovascular events, and is the main risk factor for the development of gout [3, 4]. Gout is a chronic and degenerative disease of the joints produced by the deposition of uric acid in the form of monosodium urate crystals (MSU) inside the joints and periarticular soft tissue [5].

MSU crystals initiate, amplify, and maintain an innate immune response because they are phagocytosed as foreign particles by macrophages through recognition by Toll-like receptors 2 and 4 (TLR2/TLR4) and subsequent activation and oligomerization of the NLRP3 complex (NALP3 or inflammasome). The latter is a multiprotein group with proteolytic activity that allows the activation of proinflammatory caspases, which transform the precursor of interleukin-1β (pre-IL1β) into the active form, i.e., IL-1β, which, when secreted in the extracellular medium, triggers acute inflammation [6]. Likewise, MSU promote the expression of other cytokines, such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interferon gamma (IFN-γ), and chemokines, such as monocyte chemoattractant protein-1 (MCP-1), which induces the recruitment of innate immune cells [7].

In addition to inflammation, MSU promote oxidative stress (OS) associated with respiratory bursts during crystal phagocytosis and the release of extracellular traps, which are generated mainly in neutrophils [8]. During these processes, various reactive oxygen species (ROS), such as superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂), and reactive nitrogen species, such as nitric oxide (NO), are produced; these molecules induce damage to the synovial membrane and other adjacent joint tissue [9, 10]. The interaction between MSU and receptors on the surface of synoviocytes, mainly fibroblast-type synoviocytes, generates OS, favoring the oxidation of proteins as well as an inflammatory state due to the increase in IL-1β, IL-6, and IL-8. Cyclooxygenase-2 (CCL-2) and TNF-α negatively impact viability and synovial morphology [11]. These physiological characteristics that MSU generate in synovial cells have promoted the use and study of antioxidant agents as therapies to improve the management and treatment of gout in recent years.

Compounds related to ferulic acid and gallic acid (GA) have been evaluated against xanthine oxidase and CCL-2, showing anti-inflammatory properties due to a reduction in TNF-α between 30 and 40% and in IL-6 of 60 to 75%, positioning them as candidates for the optimization, design, and development as drugs against gout [12]. GA is a polyphenolic compound present in plants such as oak and chestnut and in beverages such as wine and green tea, to which antioxidant and anti-inflammatory properties, among others, are attributed [13, 14]. However, GA is found in nature mainly in the form of tannins, and when isolated, it has low thermal stability and very low solubility in water (1.1 mg/ml), which sometimes hinders the development of formulations for its use in the clinic. Both GA and its commercial form of soluble sodium salt, sodium gallate (the latter is usually the most used pharmacological form), have uses as neoplastic agents, apoptosis inducers or antioxidants, among others. However, it is not very stable in many physiological media; it has the ability to penetrate cells, a property that is not always desirable in some treatments; therefore, its application is restricted for prolonged effects. Alternatively, the synthesis of poly-gallic acid (PGAL), through the poly-oxidation of GA mediated by the laccase of Trametes versicolor in aqueous medium [15], allowed obtaining a highly water-soluble, cytoprotective compound in fibroblasts exposed to UV radiation and with antioxidant [16] and anti-inflammatory properties in vitro models of inflammation through the activation of the protein kinase C (PKC) pathway [17, 18]. Therefore, it is important to investigate whether these properties can be considered therapeutic in diseases that occur through oxidation and inflammation mechanisms, such as gout. Derived from the above, the objective of this study was to evaluate and characterize the properties of PGAL as an inhibitor of ROS formation, inflammation, and phagocytosis in an in vitro model of gout.

MATERIALS AND METHODS

Isolation, Cell Culture, and Phenotyping of Synoviocytes

After obtaining informed consent, synovial samples (SSs) were collected from patients undergoing knee joint replacement for grade III (60%) and IV (40%)
osteoarthritis (OA) at the Instituto Nacional de Rehabilitación “Luis Guillermo Ibarra-Ibarra” (INR-LGII). Primary synoviocyte cultures were performed by mechanoenzymatic disaggregation of SSs from five patients (n = 5). The patients were female (80%) and male (20%), their average age was 67 ± 4.43, and their average body mass index was 27 ± 4.52. None had history of gout or clinically evidence of rheumatoid arthritis, and their synovial fluids were not found to be inflammatory at the time of surgery.

The cells were isolated from synovial tissue by digestion with 1 mg/ml collagenase type IA (Gibco, Life Technologies) for 2 h mixing at 37 °C and viable synoviocytes were cultured and incubated at 37 °C in an atmosphere of 5% CO2 and 95% humidity in DMEM-F12 medium containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S) into T25/ flasks, one per patient. The cells were harvested at confluence; TrypLE Express (Gibco, Life Technologies) was used for passage for the experiments. Cell viability was evaluated using trypan blue dye. After the third passage, 500,000 synoviocytes were phenotyping of fibroblast-type synoviocytes by expression of prolyl-hydroxylase (PHD1) by immunofluorescence (IF) assay and western blot [11]. For the IF, cells were seeded into fixed and permeated. Subsequently, cells were incubated with recombinant anti-PHD1/prolyl hydroxylase antibody 1:100 (ab108980, Abcam), followed by a secondary antibody 1:200 Alexa Fluor 488 (ab150073, Abcam). Images were captured with an Evos Floid microscope, Thermo Scientific (Supplementary file 1). Other fibroblast-type synoviocytes were cultured to evaluate the effects of different doses of PGAL for 24 h. This protocol was approved by a research committee (registration number 27/20).

**Effect of PGAL on Synovial Cytotoxicity**

PGAL was synthesized as reported in Zamudio-Cuevas et al. [18]; the product had an average molecular weight of 7,000 Da, based on SEC analysis, and its molecular structure was corroborated by NMR and FT-IR analysis [15]. A total of 150,000 cells were cultured per well in 12-well plates and incubated at 37 °C in an atmosphere of 5% CO2 and 95% humidity for 24 h using complete medium. To stimulate the synoviocytes with different doses of PGAL, the medium was replaced with DMEM-F12 containing 2.5% FBS and 1% P/S. To assess the effect of PGAL on cell viability, morphological changes were evaluated by crystal violet staining followed by microscopy and an assessment of stain absorbance using a plate reader. Cells exposed to different concentrations of PGAL for 24 h were fixed with 2.5% glutaraldehyde (Hycel) for 10 min and stained with 0.1% crystal violet (Sigma–Aldrich) to quantify the percentage of live cells [19] and to establish the working dose. Cells that were not stimulated were used as the control to normalize the data analysis, considering the absorbance of the control as 100% viable cells [20].

**Synovial Stimulation with MSU Crystals and PGAL**

The experimental design was based on the activation of 250,000 synovial cells with MSU crystals (100 µg/ml). MSU crystals were synthesized and were sterilized at 180 °C for 2 h according to Zamudio-Cuevas et al. [10]. The absence of microbial contaminants was confirmed by negative cultures for microorganisms using blood culture bottles from the Bactec System (Becton Dickinson) and was bacterial endotoxin-free. The synovial stimulation with MSU crystals was in DMEM-F12 culture medium containing 2.5% FBS and 1% P/S with incubation at 37 °C in an atmosphere of 5% CO2 and 95% humidity for 24 h. Other groups of cells were exposed to 100 and 200 µg/ml PGAL plus MSU crystals for 24 h. Cells cultured without MSU crystals and without PGAL were used as a negative control. Additionally, synoviocytes stimulated with 100 and 200 µg/ml PGAL were used as vehicle controls.

**Determination of IL-1β**

IL-1β was quantified with a Human IL-1β Standard ABTS ELISA Development Kit (Cat # 900-K95; Peprotech). One hundred microliters of supernatant from the synovial stimulation experiments with MSU crystals and PGAL was placed in a microplate previously coated for 24 h with the anti-IL-1β capture antibody. The cell supernatants were incubated for 2 h, and each well was washed with phosphate buffer (PBS/Tween). The detection antibody was added, and the plates were incubated for 2 h at room temperature. Finally, avidin-HRP and ABTS substrate solution were added, and the plate was incubated for 30 min. The absorbance was measured at 405 nm. The results obtained were compared to a standard curve of IL-1β and are reported in pg/ml.
Analysis of Intracellular Oxidative Stress

Intracellular ROS were quantified with CellROX fluorogenic reagent (Molecular Probes, Life Technologies, Cat. C10422®), which is designed to measure ROS in living cells. After oxidation, CellROX exhibits a signal at 640/665 nm. After the synoviocytes were treated, they were disaggregated, and 5 µM CellROX was added to each well. Then, the cells were incubated for 30 min at 37 °C protected from light. Next, the cells were washed 3 times with PBS, and ROS were quantified by image-based cytometry in a Tali image-based cytometer (Invitrogen, Life Technologies®). The data analysis was based on the fluorescence of the cells exposed to different treatments; the percentage of cells with ROS, in arbitrary fluorescence units (AFU), was normalized to the control group.

Determination of MSU Crystal Phagocytosis

The effect of PGAL on MSU phagocytosis was analyzed through the internalization of MSU crystals by cells. Synoviocytes were stimulated with PGAL at 100 or 200 µg/ml as well as with MSU for 24, 48, or 72 h. The internalization of MSU crystals was identified through the disaggregation of cultures with trypsin–EDTA (Gibco) and the subsequent visualization of the cells using polarized and compensated light microscopy (Axioscope, Carl Zeiss). The percentage of synoviocytes with at least one MSU inside was calculated based on the total number of cells examined (100 cells) and expressed as a phagocytosis index (PI) [20].

Identification of Synovial Vesicles Generated by MSU Crystals

The number of vesicles generated in synoviocytes exposed to PGAL and MSU crystals for 24 h was evaluated through morphological changes in the cytoplasm identified as “cellular orifices.” The cells were fixed with 2.5% glutaraldehyde for 15 min at 4 °C. Subsequently, staining was performed with 0.1% crystal violet, and the cells were observed under a microscope (Axioscope, Carl Zeiss). At least 4 fields were documented for each treatment evaluated, and ImageJ was used for the quantitative analysis of the vesicles.

Statistical Analysis

All assays were performed in triplicate with cells from at least 5 different patients. The results were grouped for analysis of variance (ANOVA) with Tukey’s post hoc test. p < 0.05 was used to identify significant differences. Prism V.9.1.2 (255) software (GraphPad Prism) was used.

RESULTS

Effect of PGAL on Synovial Viability and Death

No morphological changes suggestive of cellular damage were observed in synoviocytes treated with PGAL (1–500 µg/ml) for 24 h (Fig. 1A–E). Incubation with 200 and 500 µg/ml PGAL resulted in slight decreases in synovial viability of 10 and 17%, respectively; however, the decreases were not statistically significant (Fig. 1G).

Effect of PGAL on MSU Crystal-Induced Cytotoxicity

To determine the effect of PGAL on MSU crystal-induced cell death, synoviocytes were stimulated with 100 µg/ml MSU and with 100 or 200 µg/ml PGAL for 24 h. MSU crystals decreased cell viability by 20%, a significant difference with respect to unstimulated cells (Fig. 1B). For both doses of PGAL in cells exposed to MSU, an increase in the number of cells was observed. Interestingly, compared with MSU crystals only, 100 and 200 µg/ml PGAL significantly reduced cell death by 30% and 17%, respectively. Additionally, the vehicle (PGAL) alone did not induce cell death (Fig. 2).

Effect of PGAL on MSU Crystal-Induced Synovial Inflammation

To analyze the anti-inflammatory effect of PGAL, synoviocytes were incubated with MSU crystals in the presence or absence of PGAL for 24 h. As shown in Fig. 3, MSU crystals significantly increased the production of IL-1β (514.66 ± 182.82 pg/ml); however, IL-1β was inhibited when PGAL was added to the culture. For 100 µg/ml PGAL, there was a decrease of 342 ± 243.24 pg/ml, and 200 µg/ml PGAL significantly reduced IL-1β production. Cells with PGAL 100 µg/ml show a release of 85 ± 125 pg/ml of IL-1β, and PGAL 200 µg/ml produced higher IL-1β than those generated in the controls (137 ± 120 pg/ml); however, these increases are not statistically significant with respect to the control.
Fig. 1 Effect of PGAL on synovial viability. PGAL does not have a cytotoxic effect on synovial cultures. A Control, B 1 µg/ml PGAL, C 10 µg/ml PGAL, D 100 µg/ml PGAL, E 200 µg/ml PGAL, and F 500 µg/ml PGAL. Images were obtained with a 20× objective; scale bar is 100 µm (ordinary light microscopy). Images are representative of 1 of 5 independent experiments. G Quantification of the viability of synoviocytes treated with PGAL (24 h) by spectrophotometry. The values are expressed as the mean ± standard deviation.
Fig. 2 Effect of PGAL on the viability of human synoviocytes exposed to MSU. PGAL increases the viability of synoviocytes exposed to MSU crystals. The graph shows the quantification of the viability of cells exposed to MSU crystals and PGAL (24 h). Values are expressed as the mean ± standard deviation. *$p<0.05$, ***$p<0.001$.

Fig. 3 PGAL decreases the expression of IL-1β in human synoviocytes exposed to MSU. The graph shows the decrease in IL-1β induced by MSU crystals in cells treated with PGAL. Extracellular IL-1β was quantified by ELISA. Values are expressed as the mean ± standard deviation. *$p<0.05$. 
Effect of PGAL on the Inhibition of ROS Induced by MSU Crystals in Synoviocytes

Compared with cells incubated with MSU crystals but without PGAL (1.9 AFU), the addition of 100 μg/ml PGAL to synoviocytes incubated with MSU crystals decreased ROS (1.28 AFU); 200 μg/ml PGAL also significantly decreased ROS (1.46 AFU). The results for PGAL alone (vehicle) were 0.5 AFU (100 μg/ml) and 0.49 AFU (200 μg/ml) with respect to the control. PGAL concentrations did not induce greater oxidation than did baseline conditions in control cells (Fig. 4).

Effect of PGAL on MSU Crystal Phagocytosis in Synoviocytes

Compared to that in cells without MSU crystals, the PI increased by 86% in cells treated with MSU crystals; however, when 100 μg/ml or 200 μg/ml PGAL was added to the cultures, the PI decreased by 37 ± 14% (p < 0.05) and 24 ± 11%, respectively, with respect to cells incubated with MSU crystals but without PGAL for 24 h (Fig. 5A). Similarly, treatment with PGAL for 48 h decreased the PI by 39 ± 3.5% and 32 ± 5% (p < 0.05), respectively (Fig. 5B), with respect to the...
PI of 83 ± 1.4% for cells incubated with MSU crystals but without PGAL. After 72 h, with respect to that for the untreated cells, the PI for cells incubated with MSU crystals was 86% (p < 0.05), but 100 μg/ml and 200 μg/ml PGAL decreased the PI by 30% and 26%, respectively, compared with that for synoviocytes stimulated with MSU but not incubated with PGAL (data not shown).

**Effect of PGAL on the Formation of Synovial Vesicles Generated by MSU Crystals**

Multiple vesicles were observed in synoviocytes exposed to MSU crystals (172 ± 38) at 24 h, significantly more than observed in the controls (8 ± 1). PGAL significantly reduced the number of vesicles formed in cells exposed to MSU crystals, i.e., 75 ± 40 and 40 ± 16 vesicles for 100 μg/ml and 200 μg/ml PGAL, respectively (Fig. 6).

**DISCUSSION**

Synoviocytes play a major role in gout because MSU crystals induce inflammatory, and OS associated with phagocytosis [11]. The working doses of the anti-inflammatory agent PGAL were established from viability curves at 24 h in synoviocytes stimulated with 1–500 μg/ml. In this range of concentrations, no morphological changes suggestive of cell damage or death or decreased synovial viability were identified; therefore, the doses selected were 100 and 200 μg/ml. In addition, in a recent study by our group, it was demonstrated that PGAL at these concentrations was capable of modulating IL-6, IL-8, and TNF-α in THP-1 monocytes activated with phorbol myristate acetate [18].

In the present model of synovial damage, it was possible to demonstrate that the PGAL molecule has cytoprotective properties because it significantly reduces cell death caused by MSU crystals and maintains the viability of cells exposed to these crystals. Sánchez-Sánchez et al. [16] reported protective effects of PGAL against the damage caused by UV radiation in fibroblasts, maintaining a good antioxidant capacity, which is unlikely using other polyphenols such as GA under these conditions. This effect was also shown in our model by inhibiting the generation of ROS in cells exposed to MSU crystals. In another in vitro model of gout studied by Oliviero et al. [21], THP-1 cells stimulated with MSU crystals for 24 h increased ROS production up to 5.5 times more than baseline levels. However, the use of polyphenols such as resveratrol and polydatin was effective in inhibiting ROS when they were added together with the crystals; however, the only polyphenol that facilitated a decrease in the PI was resveratrol. Resveratrol suppresses the activation of NLRP3 both in vitro and in vivo, and a possible

![Fig. 5](image-url) PGAL decreases the PI in synoviocytes exposed to MSU crystals. A The dots show the quantification of the PI in synoviocytes stimulated with MSU crystals and treated with PGAL for 24 h. B Quantification of the PI at 48 h. PI was determined using polarized and compensated light microscopy. Values are expressed as the mean ± standard deviation. *p < 0.05, **p < 0.001.
mechanism for this action is through the suppression of mitochondrial ROS [22].

The antioxidant and anti-inflammatory properties of PGAL are attributed to its chemical structure, described as a multiradical polyanion (approximately 40–60 GA units with CC bonds) in a helical structure due to repulsion between its benzene rings. The rotation of this helix on its axis establishes intramolecular and intermolecular hydrogen bonds [23]. PGAL, by having stable free radicals in its structure, exerts an antioxidant mechanism of action mainly via single electron transfer (SET) instead of the most common mode of electron transfer in polyphenols and other antioxidants, i.e., hydrogen atom transfer (HAT) [24]. Both mechanisms lead to the elimination of free radicals [25]; however, SET may have interesting implications for certain treatments. HAT is dependent on the environment, and its effectiveness is greater for protonated forms of polyphenolic acids, which can be affected by the acid–base balance under physiological conditions or at an alkaline pH. Romero-Montero et al. [17] demonstrated that the hydroxyl group of the molecule makes it particularly available for the prevention and control of cell membrane lipoperoxidation.

Fig. 6 PGAL reduces the formation of vesicles in cells stimulated with MSU crystals. Images were obtained with a 20× objective; scale bar is 100 µm. Images were captured using transmitted light microscopy. The vesicles in synoviocytes across 24 h were quantified using bright field microscopy and ImageJ software. Values are expressed as the mean ± standard deviation of at least 3 independent experiments. **p<0.05 ***p<0.001.
In addition to the ability to inhibit ROS, the poly-anionic structure in PGAL suggests a barrier mechanism that prevents the recognition of crystals by cell membrane receptors, inhibiting their phagocytosis and thus inhibiting the internalization of MSU by synovial cells. Within cells, PGAL interferes in the formation of vesicles or vacuoles, which are associated with crystal phagocytosis (Fig. 7).

These vesicles are characteristic of cells that carry out phagocytosis [26]; therefore, our results corroborate the hypothesis that PGAL interferes with this primary mechanism for the activation of inflammation in gout by inhibiting IL-1β. In addition, an in vitro study indicated that PGAL has greater hydroxyl radical capture activity than does GA and a more significant protective effect on cellular damage induced by H₂O₂ [17].

The mechanisms of inflammation and OS that MSU crystals activate in cells when recognized by TLR receptors through the plasma membrane involve NF-kB. A preliminary study indicated that PGAL reduces the expression of NF-kB (data not shown) and other components in THP-1 cells for inflammasome activation, thus inducing caspase-1 and IL-1β release, or activates gasdermin D, which promotes the formation of pores in the membrane, inducing pyroptosis [27].

Pyroptosis occurs after the intracellular detection of damage signals, which can be induced by MSU crystals. Pyroptotic cells present membrane pore formation and plasma membrane rupture and release inflammatory mediators and cytoplasmic content into the extracellular space [28, 29]. Therefore, the formation of vesicles identified in synoviocytes could be associated with the activation of pyroptosis, and in this sense, PGAL, by inhibiting the formation of these vesicles, would exert a possible mechanism of action through the inhibition of inflammasomes or of pyroptosis. Similar to its precursor, GA, which was studied in in vitro and in vivo models of gout [30], was shown to inhibit pyroptosis in macrophages stimulated by MSU crystals, block the activation of NLRP3, inhibit caspase-1 activation and IL-1β secretion, and promote the expression of factor 2 related to nuclear factor E2 (Nrf2), reducing mitochondrial ROS, which supports our hypothesis that a similar mechanism occurs for PGAL.
Our results demonstrate the antioxidant and anti-inflammatory properties of PGAL in synoviocytes exposed to MSU crystals in a model that mimics an acute attack of gout. Therefore, this research is relevant, and more studies are being carried out to elucidate the molecular pathways by which PGAL regulates phagocytic activity in cells as well as the inflammatory and oxidative states in gout. In vivo molecular studies are of interest to elucidate the mechanisms of action of PGAL in gout, in particular, those aimed at the inhibition of MSU crystals recognition in synoviocytes, and its potential therapeutic role for the treatment of this disease.

CONCLUSIONS

PGAL exhibits a cytoprotective effect against damage caused by MSU crystals in human synoviocytes and has the potential to counteract the oxidative and inflammatory responses induced by MSU crystals. The results obtained suggest that PGAL exerts action at the membrane level that prevents the phagocytosis of crystals, inhibiting the formation of synovial vesicles and decreasing IL-1β.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

YZC, RSS, and MG contributed to the conception and design, data collection, analysis, and interpretation, and writing and critical revision of the article. VML, IALJ, NMA, KFM, and JFT contributed to data analysis and interpretation as well as writing and critical revision of the article.

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DECLARATIONS

Ethics Approval The protocol was reviewed and accepted by the research committee of INR-LGII, number 27/20.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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