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# 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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**Research Article**

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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 24h 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.


# 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, resulting in 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 are the main stimuli that 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 ($O_2^-$) and hydrogen peroxide ($H_2O_2$), 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 to 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 *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 4 osteoarthritis (OA) at the National Institute of Rehabilitation. Primary synoviocyte cultures were performed by mechanoenzymatic disaggregation of SSs, and viable

synoviocytes were cultured and incubated at 37°C in an atmosphere of 5% $CO_2$ and 95% humidity in DMEM-F12 medium containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S). Cell viability was evaluated using trypan blue dye. After the third passage, 500,000 synoviocytes were immunophenotyped, and others 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% $CO_2$ 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 supplemented with 2.5% FBS, 1% P/S and PGAL (1, 10, 100, 200 and 500 µg/mL). For the control group, synoviocytes without PGAL were only cultured 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 MUCs and PGAL

The experimental design was based on the activation of 250,000 synovial cells with MSU (100 µg/mL) in DMEM-F12 culture medium containing 2.5% FBS and 1% P/S with incubation at 37°C in an atmosphere of 5% $CO_2$ and 95% humidity for 24 h. Other groups of cells were exposed to 100 and 200 µg/mL PGAL plus MSU for 24 h. Cells cultured without MSU 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 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 phagocytosis

The effect of PGAL on MSU phagocytosis was analyzed through the internalization of MSU 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 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

The number of vesicles generated in synoviocytes exposed to PGAL and MSU 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 (Figure 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 (Figure 1G).

# Effect of PGAL on MSU-induced cytotoxicity

To determine the effect of PGAL on MSU-induced cell death, synoviocytes were stimulated with 100 μg/ml MSU and with 100 or 200 μg/ml PGAL for 24 h. MSU decreased cell viability by 20%, a significant difference with respect to unstimulated cells (Figure 1B). For both doses of PGAL in cells exposed to MSU, an increase in the number of cells was observed. Interestingly, compared with MSU 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 (Figure 2).

# Effect of PGAL on MSU-induced synovial inflammation

To analyze the anti-inflammatory effect of PGAL, synoviocytes were incubated with MSU in the presence or absence of PGAL for 24 h. As shown in Figure 3, MSU 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.

# Effect of PGAL on the inhibition of ROS induced by MSU in synoviocytes

Compared with cells incubated with MSU but without PGAL (1.9 AFU), the addition of 100 μg/ml PGAL to synoviocytes incubated with MSU 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 (Figure 4).

# Effect of PGAL on MSU phagocytosis in synoviocytes

Compared to that in cells without MSU, the PI increased by 86% in cells treated with MSU; 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 but without PGAL for 24 h (Figure 5A). Similarly, treatment with PGAL for 48 h decreased the PI by 39 ± 3.5% and 32 ± 5% ($P < 0.05$), respectively (Figure 5B), with respect to the PI of 83 ± 1.4% for cells incubated with MSU but without PGAL. After 72 h, with respect to that for the untreated cells, the PI for cells incubated with MSU 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

Multiple vesicles were observed in synoviocytes exposed to MSU (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, i.e. 75± 40 and 40± 16 vesicles for 100 µg/ml and 200 µg/ml PGAL, respectively (Figure 6).

# Discussion

Synoviocytes play a major role in gout because MSU 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 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. In another *in vitro* model of gout studied by Oliviero et al. [21], THP-1 cells stimulated with MSU 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 were 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 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.

In addition to the ability to inhibit ROS, the polyanionic 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 (Figure 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 $H_2O_2$ [17].

The mechanisms of inflammation and OS that MSU 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. 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, 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 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 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 in human synoviocytes and has the potential to counteract the oxidative and inflammatory responses induced by MSU. 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.

# Declarations


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### COMPETING INTERESTS

Theauthorsdeclarethattheyhave no competing interests.

### AUTHORS' CONTRIBUTIONS

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.

### COMPLIANCE WITH ETHICAL STANDARDS

Competing Interests. The authors declare that they have no competing interests. Ethics Approval. The protocol was reviewed and accepted by the research committee of INRLGII, number 27/20.

**CONSENT FOR PUBLICATION**. Not applicable.

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# Figures

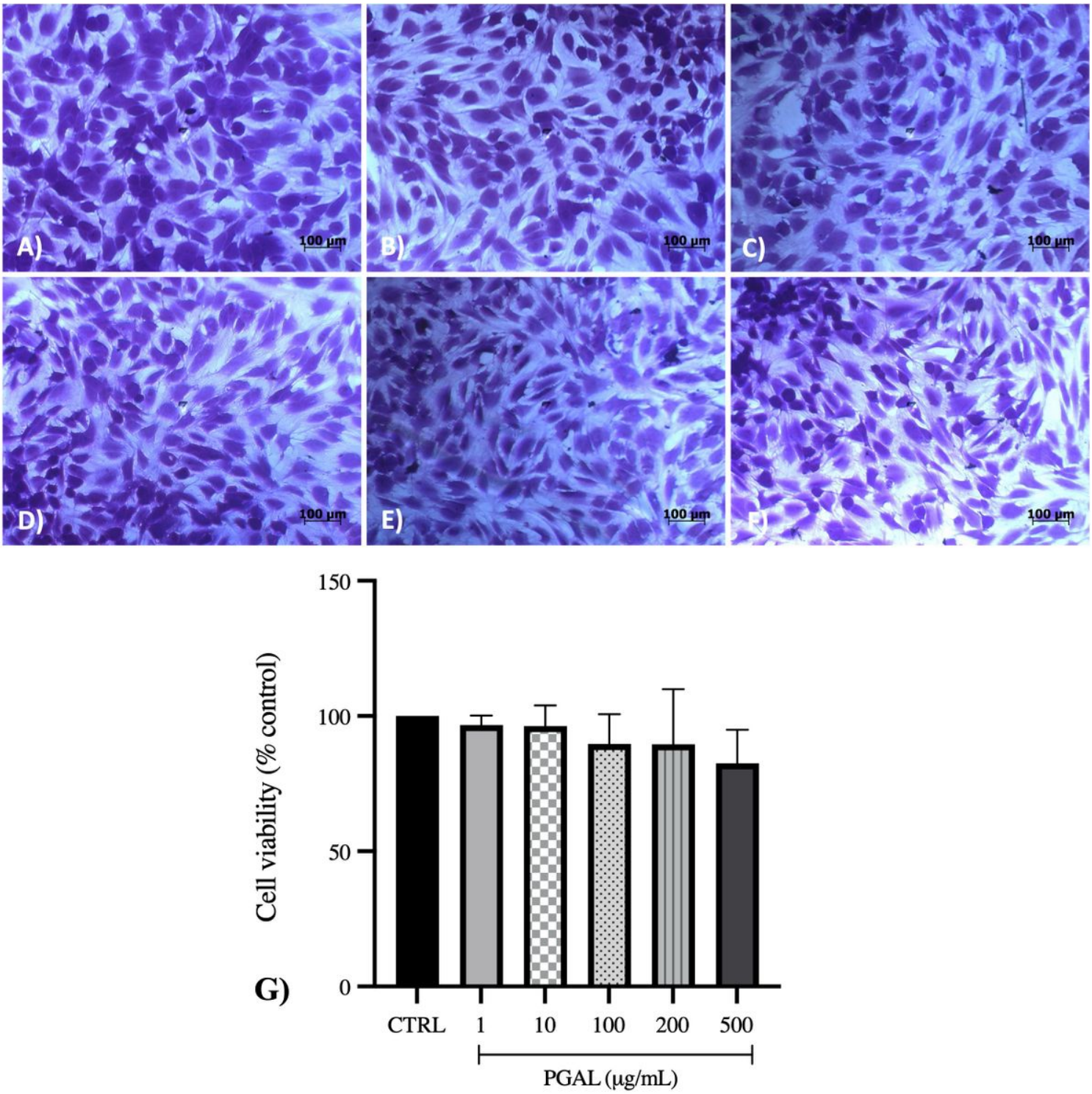
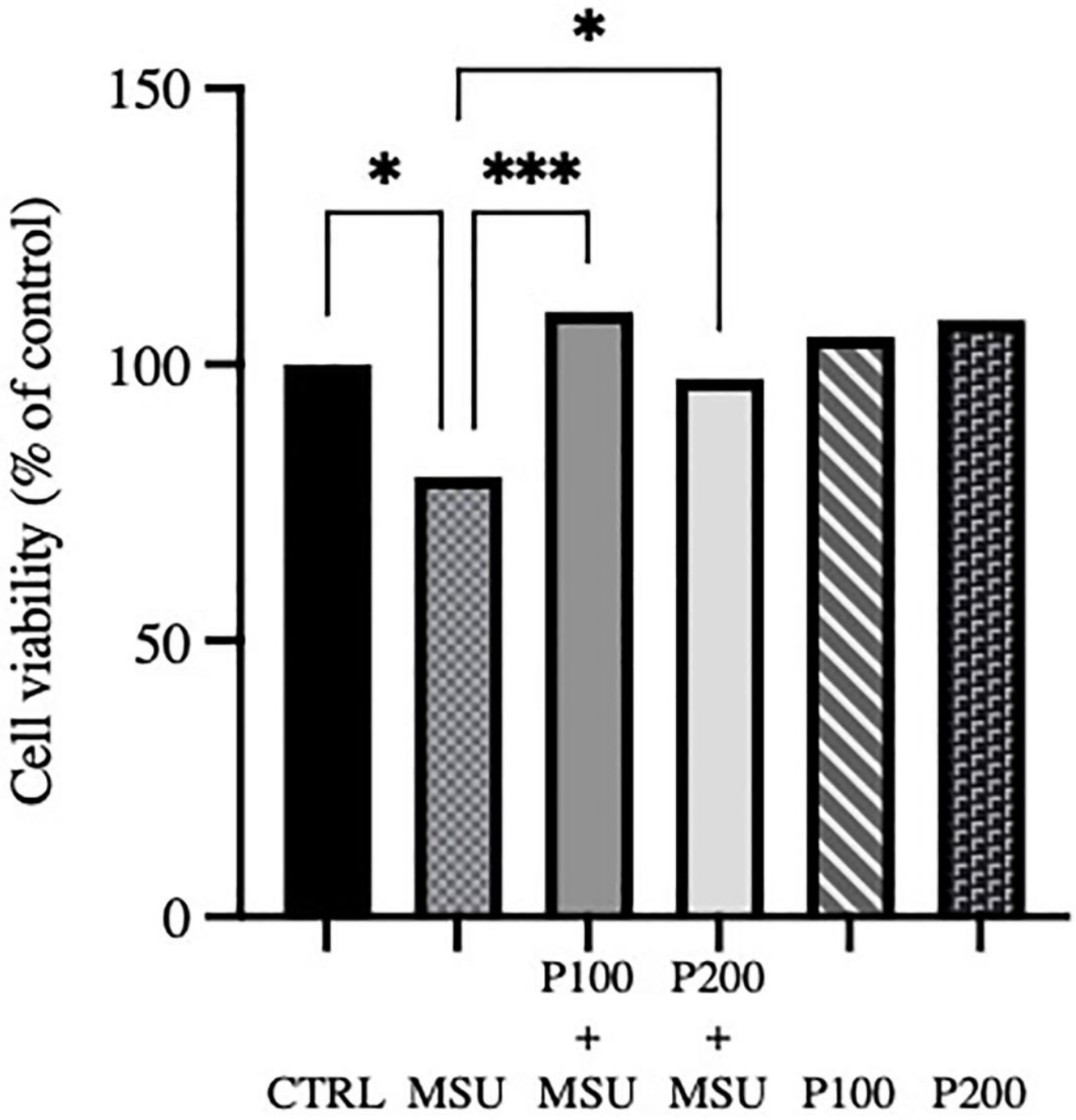



**Figure 1**

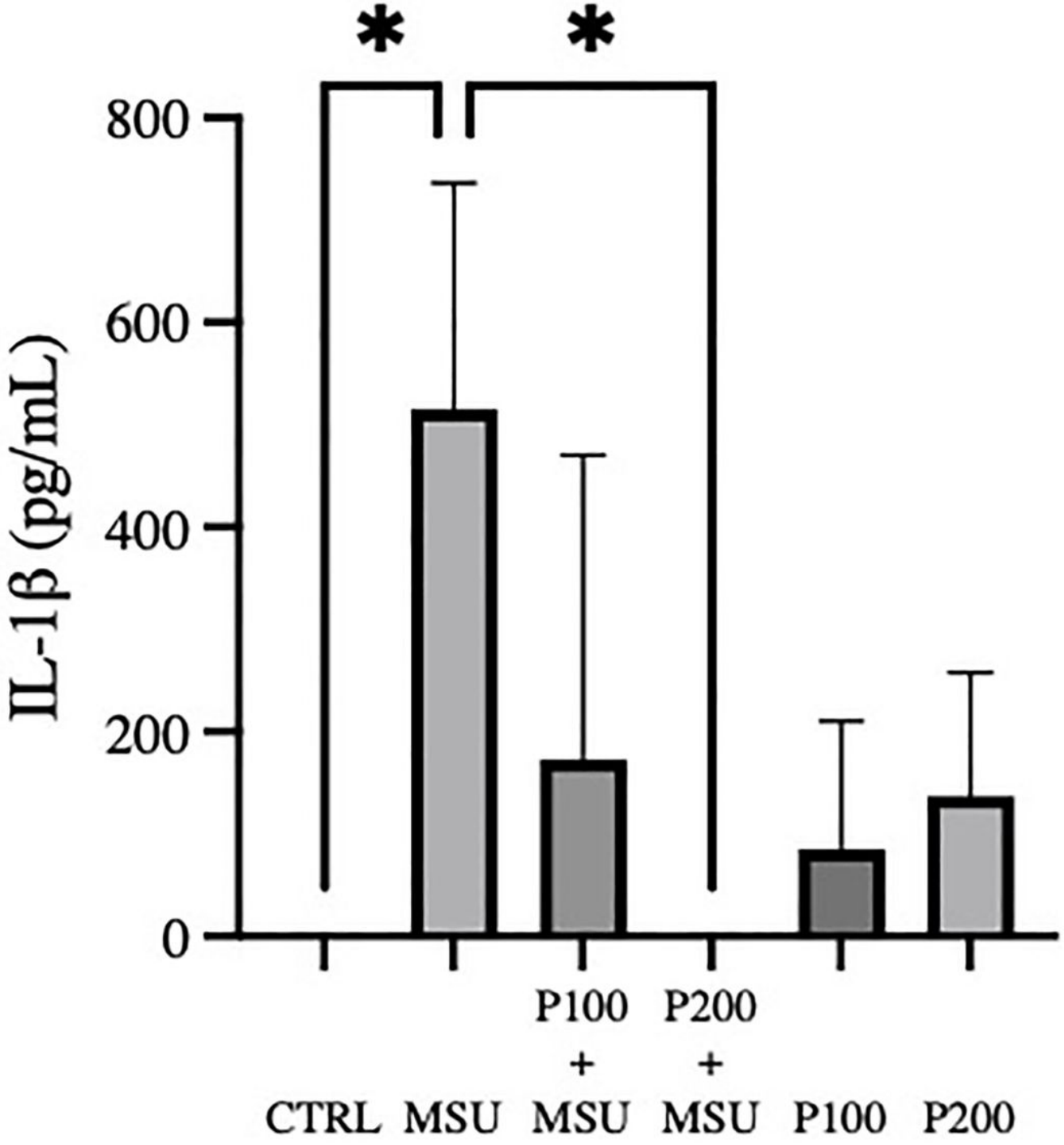
**Effect of PGAL on synovial viability**. PGAL does not have a cytotoxic effect on synovial cultures. A) Control, B) 1 mg/ml PGAL, C) 10 mg/ml PGAL, D) 100 mg/ml PGAL, E) 200 mg/ml PGAL, and F) 500 mg/ml PGAL. Images were obtained with a 20x objective; scale bar is 100 mm (ordinary light microscopy). 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.




## Figure 2

**Effect of PGAL on the viability of human synoviocytes exposed to MSU**. PGAL increases the viability of synoviocytes exposed to MSU. The graph shows the quantification of the viability of cells exposed to MSU and PGAL (24 h). Values are expressed as the mean ± standard deviation * $P < 0.05$, *** $P < 0.001$.




## Figure 3

**PGAL decreases the expression of IL-1b in human synoviocytes exposed to MSU**. The graph shows the decrease in IL-1b induced by MSU in cells treated with PGAL. Extracellular IL-1b was quantified by ELISA. Values are expressed as the mean ± standard deviation * $P < 0.05$.

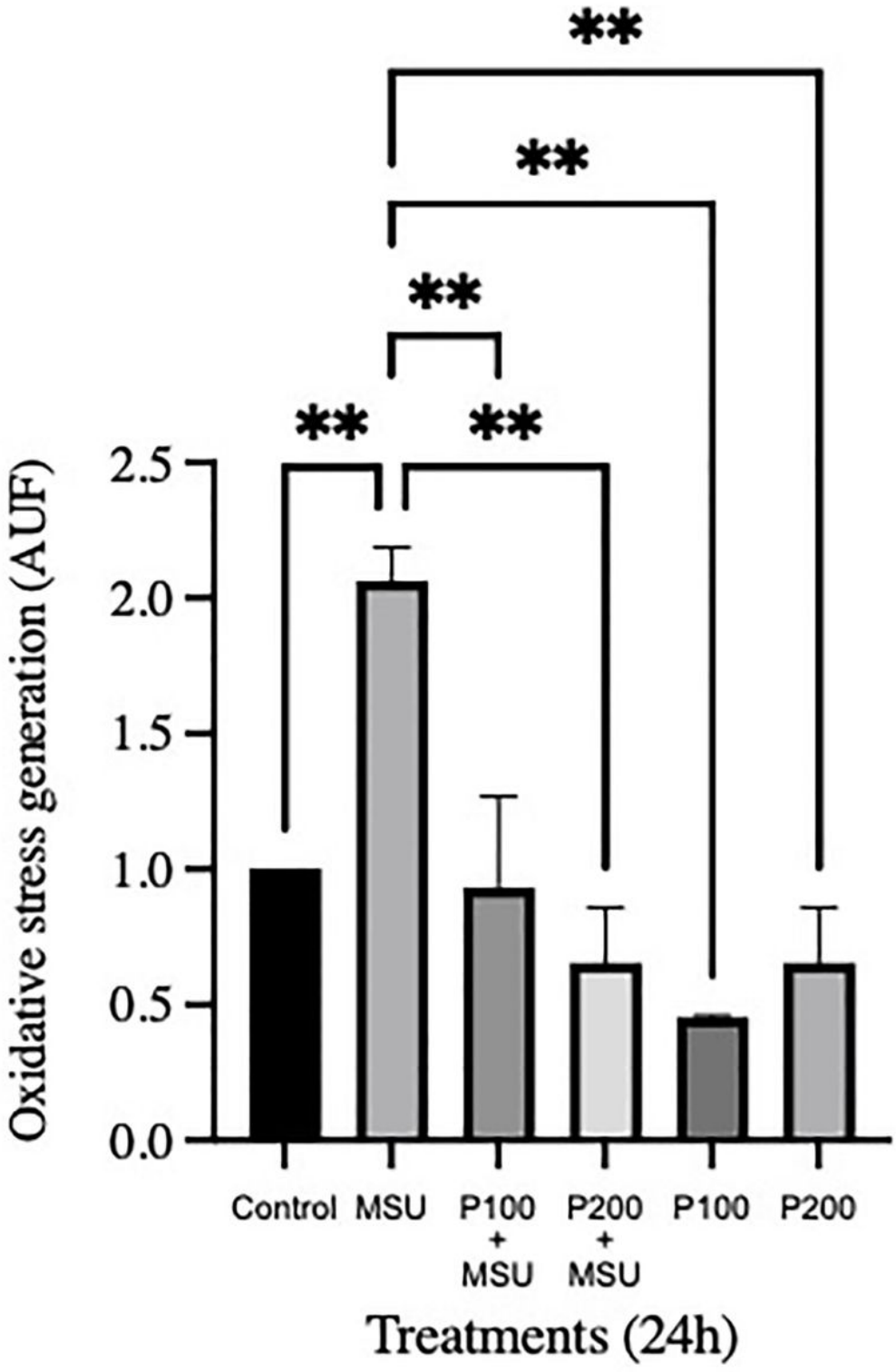
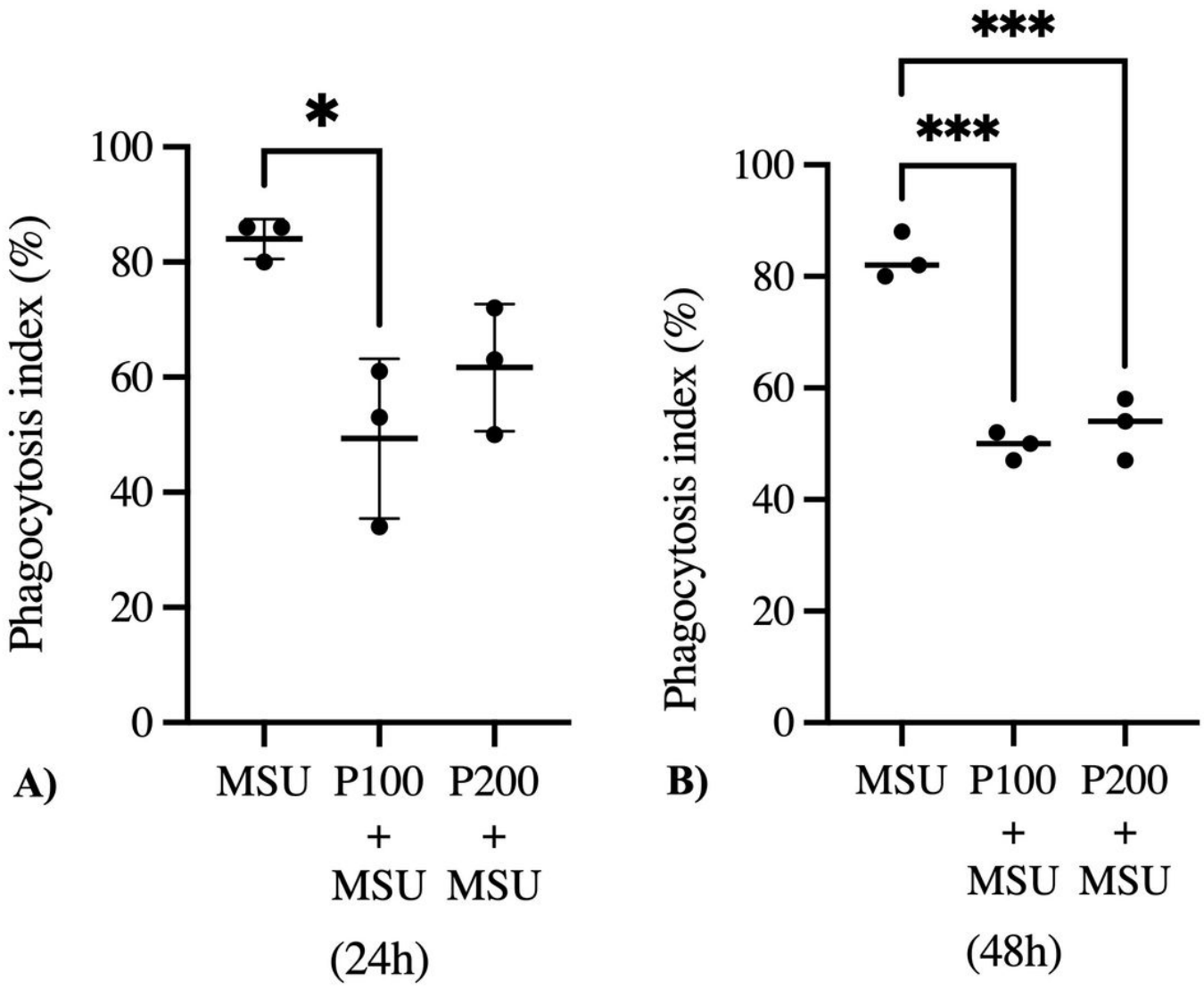



**Figure 4**

**The antioxidant effect of PGAL in synoviocytes exposed to MSU.** The graph shows the quantification of ROS in control cells, synoviocytes stimulated with MSU and synoviocytes stimulated with MSU and PGAL. The fluorescence emitted by ROS in cells was quantified by image cytometry. The data are expressed as the mean ± standard deviation. * $P < 0.05$.




## Figure 5

**PGAL decreases the PI in synoviocytes exposed to MSU**. A) The dots show the quantification of the PI in synoviocytes stimulated with MSU 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 * $P < 0.05$ *** $P < 0.001$.

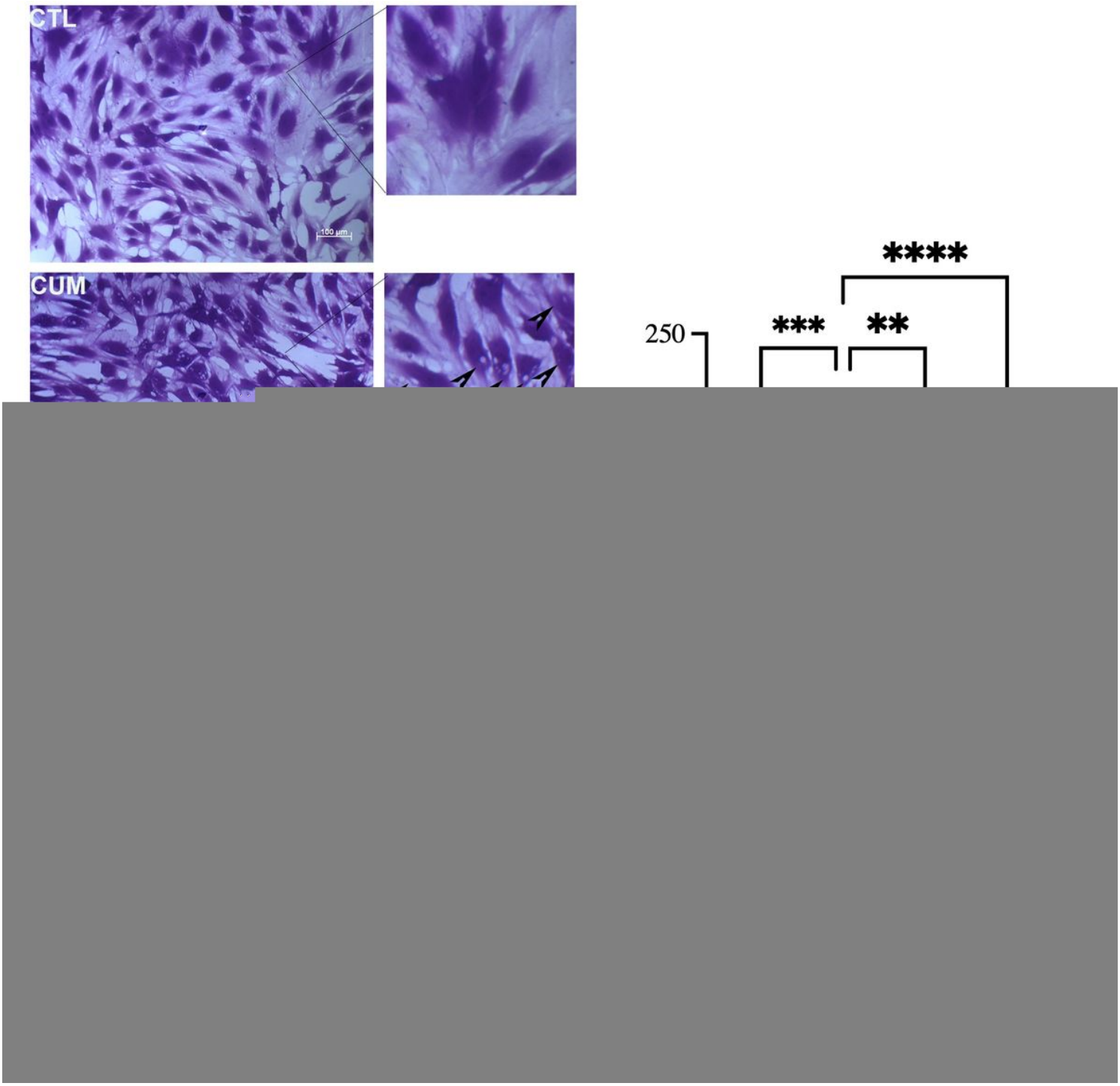



**Figure 6**

**PGAL reduces the formation of vesicles in cells stimulated with MSU**. Images were obtained with a 20x objective; scale bar is 100 mm. 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. ** $P < 0.05$ *** $P < 0.001$.

## Figure 7

**Interaction of PGAL with MSU in synoviocytes**. PGAL has a multiradical polyanion structure that establishes intramolecular and intermolecular hydrogen bonds, and the repulsion between neighboring benzenes gives a helical structure that rotates on an axis. The antioxidant capacity of PGAL is attributed to the stable free radicals in its structure; its mechanism of ROS inhibition can occur through single electron transfer (SET) or by hydrogen atom transfer (HAT). It can interact with MSU to form a complex through hydrogen bonds. This PGAL complex on the cell membrane occurs due to electrostatic interactions that prevent the passage of MSU into synoviocytes. The figure was designed using Biorender.com.