

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

DISCIPLINA DE TRABALHO DE CONCLUSÃO DE CURSO DE FARMÁCIA

VANESSA SCHUCK CLARIMUNDO

EFFECT OF RC-3095, AN ANTAGONIST OF GASTRIN-RELEASING PEPTIDE
RECEPTOR, ON FIBROBLAST-LIKE SYNOVYOCITES: A POTENTIAL NEW
STRATEGY FOR ARTHRITIS TREATMENT

Porto Alegre, dezembro de 2013.

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Trabalho de Conclusão de Curso

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COORIENTADORA: Dra. Patrícia Gnieslaw de Oliveira

Porto Alegre, dezembro de 2013.

Este trabalho foi realizado sob a forma de artigo científico a ser submetido no periódico “Annals of Rheumatic Diseases”, cujas normas técnicas encontram-se no Anexo I deste trabalho.

Effect of RC-3095, an antagonist of gastrin-releasing peptide receptor, on fibroblast-like synovocytes: a potential new strategy for arthritis treatment

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ABSTRACT

Introduction: Gastrin-releasing peptide (GRP) is a functional homologue of bombesin, and its receptor signaling is involved in several functions, including inflammatory response. GRP and its receptor (GRPR) have been found in synovial membrane and fluid of rheumatoid arthritis patients. RC-3095 is an antagonist of GRPR. **Objective:** evaluate the role of gastrin-releasing peptide, its receptor and RC-3095 on fibroblast-like synoviocytes (FLS) in RA development. **Methods:** Mouse DBA/1J FLS were isolated from the tarsus of the hind paws of collagen-induced arthritis. FLS immunocytochemistry was executed to evaluate the GRPR presence. Then, it was evaluated FLS viability treated with RC-3095 in 24 h and 48 h. After this, FLS proliferation stimulated with LPS or GRP was performed by MTT assay in 24h. Finally, invasion assay was realized in a transwell system using Matrigel-coated inserts and treated with GRP, RC-3095, GRP+RC-3095. **Results:** Immunocytochemistry confirmed the presence of GRPR in FLS and its antagonist was not cytotoxic for FLS. Any statistical difference was observed in proliferation assay. Although, in invasion assay GRP was able to increase FLS invasion and RC-3095 or GRP+RC-3095 treatment comparing with GRP was able to decrease FLS invasion. **Conclusion:** This is the first study to identify the presence of GRPR in FLS. GRP causes an increase in the FLS invasion and RC-3095 was able to reverse this effect. Therefore the interference on GRP pathway is a potential target for the treatment of rheumatoid arthritis using RC-3095 for future clinical trials.

Key Words: Gastrin-releasing peptide receptor, RC-3095, Fibroblasts like-synoviocyte, Rheumatoid arthritis, Treatment.

INTRODUCTION

Rheumatoid arthritis (RA) is considered a chronic, systemic, inflammatory and autoimmune disorder.[1-3] Worldwide RA achieve approximately 0,5% to 1% of the people, affecting more women than men.[4] It is known that the pathophysiology of this disease is an inflammation of the synovium followed by the development of a *pannus*, that leads to a destruction of the cartilage and the bone.[5] However, the etiology of this disease is still not completely understood,[5] but fibroblast-like synoviocytes [6] and neuropeptides have been suggested to play an important role in the pathogenesis of RA.[3]

The lining layer of the synovium is populated by macrophages and fibroblast-like synoviocytes (FLS), these contribute with the dynamic and structural integrity of diarthrodial joints. The fibroblasts are involved in the secretion of the components contained in the synovial fluid as lubricin and hyaluronic acid. Moreover, produce collagen, glycosaminoglycans, metalloproteinases, metalloproteinase inhibitors, cytokines, reticular fibers and glycoproteins found in the extracellular matrix.[7]

The expression of adhesion molecules on the surface of synovial fibroblasts probably directs the passage of some populations of cells, such as neutrophils into the synovial fluid and retaining other in the synovial membrane and mononuclear leukocytes. Metalloproteinases, cytokines, adhesion molecules and other cell surface molecules are deregulated in inflammatory states. Tissue damage stimulates fibroblasts and induces mitosis.[7] Nevertheless, in RA, FLS became a pathogenic factor. They play a central role in the formation of pannus, since there is a reduction in the ability of FLS undergo apoptosis, consequently, they become the majority population of cells in the synovium of patients with RA. Furthermore, they can contribute with the invasion and destruction of cartilage and bone by the production of cytokine, mediators of inflammation and proteolytic enzymes that degrade the extracellular matrix.[6]

In chronic inflammatory diseases, has been related that occur a disruption of neuroendocrine-immune interactions. The interaction between the nervous and immune system is mediated by neuropeptides and their receptors, hence these can contribute in the pathogenesis of chronic inflammation.[8] Recent studies shown that inflammatory cells can produced neuropeptides in response to a pathology, while in other situations this may induce production of cytokine by cells of the immune system.[9] Besides,

neuropeptides are involved in modulation of plasma extravasation, edema, recruitment and proliferation of immune cells.[10]

Substance P, neuropeptide Y, vasoactive intestinal peptide, neuromedin U are examples of neuropeptides that had their involvement in RA investigated. Another neuropeptide that has been reported is gastrin-releasing peptide (GRP), a functional homolog of bombesin (BN), which is a tetradecapeptide originally isolated from frog skin.[11]

GRP effects, like gastrointestinal secretion, cell proliferation and neuroendocrine regulation are mediated by gastrin-releasing peptide receptor (GRPR), a member of the G protein coupled receptors.[12] GRP plays an important role in development and regulation of the immune response by acting in immune cells that presenting GRPR as lymphocytes, neutrophils, macrophages, endothelial cells.[13, 14] In RA, specifically, there are evidences that GRP is found in murine chondrocytes [15] and in joint fluid of patients where show increased concentrations.[3] Also, it was reported that there is a correlation between some proinflammatory cytokines and GRP in joint fluid and this fact may influence the development of the disease.[3] Most interesting is the finding that synovial cells (fibroblasts and intersitial cells) or cartilage cells (chondrocytes lining the articular surface) express GRPR in experimental arthritis.[11] These observations suggest a large involvement of GRP/GRPR in RA, so this might be worthwhile in treatment of RA.

There are several antagonists of GRPR one of these is RC-3095 – the synthetic bombesin pseudonapeptides. It has been reported in the literature that RC-3095 has higher specificity on GRPR.[16] RC-3095 was able to reduce the secretion of TNF- α and IL-1 β (proinflammatory cytokines) in an experimental model of intestinal sepsis and acute lung injury.[17, 18] Recently, our group demonstrated promising results indicating the antiinflammatory effect of RC-3095 in two arthritis experimental models. RC-3095 was able to decrease joint damage and serum levels of IFN- γ , IL-1 β , TNF- α , IL-6 and IL-10 in a CIA arthritis model.[19] In this context an antagonist of GRPR could modulate the inflammatory response in RA. Based on these observations, the present study was designed to evaluate the role of gastrin-releasing peptide, its receptor and RC-3095 on FLS in RA development.

MATERIALS AND METHODS

Isolation and Culture of FLS

Mouse DBA/1J FLS were isolated from the tarsus of the hind paws of animals with collagen-induced arthritis (CIA). The assay was performed according to Laragione et al with modifications.[20] Briefly, tissues were chopped off and then were incubated for 1 h in collagenase (type IA) solution (1 mg/ml) (Sigma-Aldrich). The supernatant was collected and centrifuged at 1100 rpm for 10 minutes in room temperature. After this, the pellet was resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) containing 15% fetal bovine serum (FBS; Invitrogen) 1% of penicillin/streptomycin, 0,125% of gentamicin, 0,04% of fungizone and then the cells were transferred to a culture plate. After 24 h, adherent cells were cultured and nonadherent cells were withdrawn. The cells were kept at a temperature of 37 °C, a minimum relative humidity of 95% and an atmosphere of 5% CO₂. All the experiments of this study were conducted after FLS reached passage 4 (>95% FLS purity).

Immunofluorescence Microscopy

FLS were cultured on cover slips to a 10% to 20% confluence in complete media for 48 h. Immunofluorescence was performed using a previously reported method with modifications.[21] Summarizing, cells were fixed in 4% formaldehyde for 15 min at room temperature, then they were permeabilized with phosphate-buffered saline (PBS) plus 0,2% Triton-X100 followed by addition of 0,3% albumin in 0,2% PBS-Triton X-100 for 60 min to prevent nonspecific binding. Subsequently, samples were incubated with anti-GRPR (1:100) for 1 h at room temperature protected from light. After extensive washing with PBS, cells were incubated for 1 h with the secondary antibody anti-rabbit (1:100) marked with Cy3. Stained cells were again washed with PBS mounted under glass coverslips. For visualization of the GRPR a fluorescent microscope with appropriate filters was used.

FLS Viability Assay

Cell viability was assessed by dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (MTT) assay (Sigma). FLS were cultured in triplicate at a density of 5×10^4 /well in 96-well plate in DMEM and treated with different concentrations of RC-3095 (10 uM – 0,05 uM) for 24 h and 48 h. At the end of this period, MTT (5 mg/mL) was added to the medium and the plate was incubated for 4h at 37°C. Then, formazan crystals were

solubilized with DMSO (Sigma) and the product were quantified. The absorbance was measured at 570 nm with an ELISA plate reader (Anthos Zenyth 200rt).

Cell Proliferation Assay

FLS were cultured in 96-well plates at a density of 2×10^4 /well in DMEM and the cell proliferation was evaluated by MTT assay (Sigma). Cells were seeded and treated with LPS (1 to 10 ug/mL) or GRP (0,1 to 10 uM). After 24 h, MTT (5 mg/mL) was added to the medium and the plate was returned to the incubator for 4h. Then, the supernatants were removed and 100 uL of DMSO (Sigma) was added. The amount of formazan crystals was quantified by measuring absorbance with an ELISA plate reader (Anthos Zenyth 200rt) at a 570 nm.[11]

Invasion Assay

The invasion assay was realized using a Matrigel-coated inserts BD Bioscience (Franklin Lakes, NJ, USA) as previously described.[20, 22] Initially, the transwells were preincubated with free-serum DMEM for 2 h at 37°C. After this, FLS were plated in upper compartment in a concentration of 2×10^5 /well in 500 uL of serum-free DMEM. At the lower compartment of the insert was added DMEM with 15% FBS. Right after, RC-3095 (1 uM), GRP (10 uM), GRP (10 uM)+RC-3095 (1 uM) were added in the upper compartment of the insert. After 24 h incubation at 37°C, the supernatant was removed and the cells were stained with crystal violet (Sigma) for 20 minutes. Finally, the upper surface of the insert was cleaned with a cotton swabs to remove noninvading cells and matrigel layer. The total number of cells that invaded trough Matrigel was counted at 100x magnification. (n=4)

Statistical analysis

Data are presented as mean or mean \pm SEM. Groups were compared by the analysis of variance with Tukey's adjustment for multiple comparisons using GraphPad Prism 5.0. Statistical differences were considered to be significant when $P < 0.05$.

RESULTS

Expression of GRPR in FLS

The detection of GRPR in FLS was determined by indirect immunofluorescence microscopy using specific antibody anti-GRP (1:100). It was observed that the immunoreactivity for FLS assumed a punctiform characteristic aspect. This result shows that GRPR is present in FLS of mouse DBA/1J with CIA.

Insert Figure 1

Role of RC-3095 on FLS viability

The cytotoxicity of RC-3095 on FLS was evaluated by cell viability assay, in which cells were treated with RC-3095 at concentrations of 0.05 μM to 10 μM . We observed that RC-3095 is not cytotoxic to FLS in all concentrations tested, except at the highest concentration (10 μM) given that we can observe a decrease in cell viability by 38% after 24 h and 32% after 48 h exposure to treatment compared to control. Furthermore, we note that RC-3095 at concentration 1 μM showed a similar behavior to the control, so the dose of 1 μM was chosen to carry out the following experiments.

Insert Figure 2

GRP does not contribute with FLS proliferation

Proliferation assay was performed to evaluate the effect of GRP (0.1 – 10 μM) and LPS (1 and 10 μM) on FLS. There was not observed any statistical difference between control and treatments. However, it is notable that GRP 10 μM tends to cause an increase in proliferation, since it causes an increase in the number of cells when compared to the control and LPS, a specific stimulator of proliferation through toll-like receptor 4.

Insert Figure 3

GRP and RC-3095 affect invasion of FLS

The number of invading cells after exposure to GRP (10 μM), RC-3095 (1 μM) or GRP+RC-3095 was determined by invasion assay. Comparing with the control it can be observed that treatment with RC-3095 do not affect FLS invasion, however, when FLS were exposed to GRP there is an increased of 67% in FLS invasion ($p < 0.05$). Moreover, comparing FLS treated with RC-3095 to GRP it can be consider reduction of

65% in FLS invasion ($p < 0.01$). Lastly, there is a decrease of 45% in the FLS invasion comparing FLS treated with GRP+RC-3095 to GRP ($p < 0.001$) (Figure 4; $n=4$).

Insert Figure 4

DISCUSSION

In this study we evaluated the involvement of GRP its receptor and RC-3095 on FLS. It has been previously reported that GRP/GRPR signaling present action on the development and regulation of the immune response through various immune cells such as macrophages, mast cells, lymphocytes, neutrophils.[13, 14] In previous studies the expression of GRPR has been observed in joint on inflammatory infiltrate and articular chondrocytes in arthritic mice.[15] Additionally, it was mentioned the expression of GRP and GRPR in cells of synovial tissue of patients.[23] An important observation on this study is the finding of marked GRPR immunoreactions in FLS. This finding is significant, since GRP and its receptor may be intimately involved in the inflammatory response and consequently with the development of RA.

Many antagonists have been synthesized and tested against the inhibition of this metabolic pathway. RC-3095, a competitive antagonist of GRPR, was able to improve survival rates by reducing the inflammatory infiltrate in models of experimental sepsis and acute lung injury. Furthermore, this molecule also was capable to modulate the release of proinflammatory cytokines (TNF- α and IL-1 β) in activated macrophages in sepsis model.[18] In a model of ulcerative colitis RC-3095 reduced the severity of inflammatory bowel disease and their antiinflammatory activity was associated with a reduction of the expression of colonic TNF α . [24] In addition, RC-3095 was effective to attenuate the synovial inflammation, synovial hyperplasia, pannus formation and erosion of joint in experimental models of arthritis.[11] Because RC-3095 may represent a possible target for the pharmacological control of inflammatory response we tested it as a possible new antirheumatic drug. In our study, we observe that the RC-3095 at concentrations tested has not present any toxicity to FLS, except at the highest concentration.

In parallel, FLS have a tumor phenotype which is not observed in other types of fibroblasts, suffer a exacerbated proliferation, exhibit invasive characteristics and

resistance to undergo apoptosis.[25] So the control of FLS proliferation and invasion perform are an important therapeutic aim.

GRP is considered a mitogenic agent inducing the proliferation and cellular growth, furthermore has been demonstrated that GRP can cause a proliferation of mast cells [26] and lymphocytes.[27] In this study, we do not observe any stimulation of proliferation on FLS by GRP at tested concentrations.

On the other hand, our study demonstrated that GRP and RC-3095 were able to modulate the FLS invasion. GRP increase significantly the FLS invasion, hence we can suggest that the pathway of GRP can be involved with the development of RA by fibroblast stimulation. Moreover, we observed a significant reduction in the FLS invasion when treated with GRP+RC-3095 to GRP. Therefore, RC-3095 was able to reverse the effect over FLS. These results are interesting because it has been reported in the literature a correlation between *in vitro* invasive properties of FLS from patients with RA and from rats with pristane-induced arthritis with radiographic erosive changes and histological joint damage, respectively.[28]

CONCLUSION

This is the first study to identify the presence of the GRP receptor in FLS, a pathogenic factor in RA. We show that GRP has no effect on cell proliferation, but causes an increase of FLS migration. RC-3095 was able to reverse the effect of GRP over FLS. RC-3095 has higher specificity on GRPR and this could be preventing the action of increased levels of GRP during the inflammatory response of arthritis. This could be the motive that it is able to attenuate the development of arthritis in two arthritis experimental models and reduce joint damage, previously demonstrated by our research group. Therefore the interference on this pathway is a potential target for the treatment of rheumatoid arthritis using RC-3095 for future clinical trials.

ACKNOWLEDGEMENTS

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LEGENDS

Figure 1: Evaluation of the expression of the GRP receptor through immunocytochemistry staining in FLS of mouse DBA/1J with CIA. The cells present red fluorescence indicating the presence GRPR.

Figure 2: Assessment of cytotoxicity of RC-3095 on FLS. Cells were treated for 24 h and 48 h with the GRP antagonist, after this period the MTT assay was performed. Results are expressed as mean (%) and the percentage of viable cell was calculated by ANOVA two way followed by Tukey's adjustment.

Figure 3: Effect of GRP on the FLS proliferation. Cells were treated for 24 h with GRP (0,1 μ M – 10 μ M) and LPS (1 μ M and 10 μ M), after this period MTT assay was realized. Results are expressed as mean (%) and the statistical was calculated by ANOVA one way followed by Tukey's adjustment.

Figure 4: Involvement of RC-3095, GRP or RC-3095+GRP in invasion assay of FLS.
a) Light microscopy migration/invasion of fibroblasts stained with crystal violet, 100X.
b) Cell invasion for 24 h was assessed by matrigel invasion assay. Cells were treated with 1 μ M RC-3095, 10 μ M GRP or both. Bars represent mean \pm SEM; (*) $p < 0.05$ GRP x control; (##) $p < 0.01$ GRP x GRP+RC-3095; (###) $p < 0,001$ GRP x RC-3095.

FIGURE 1

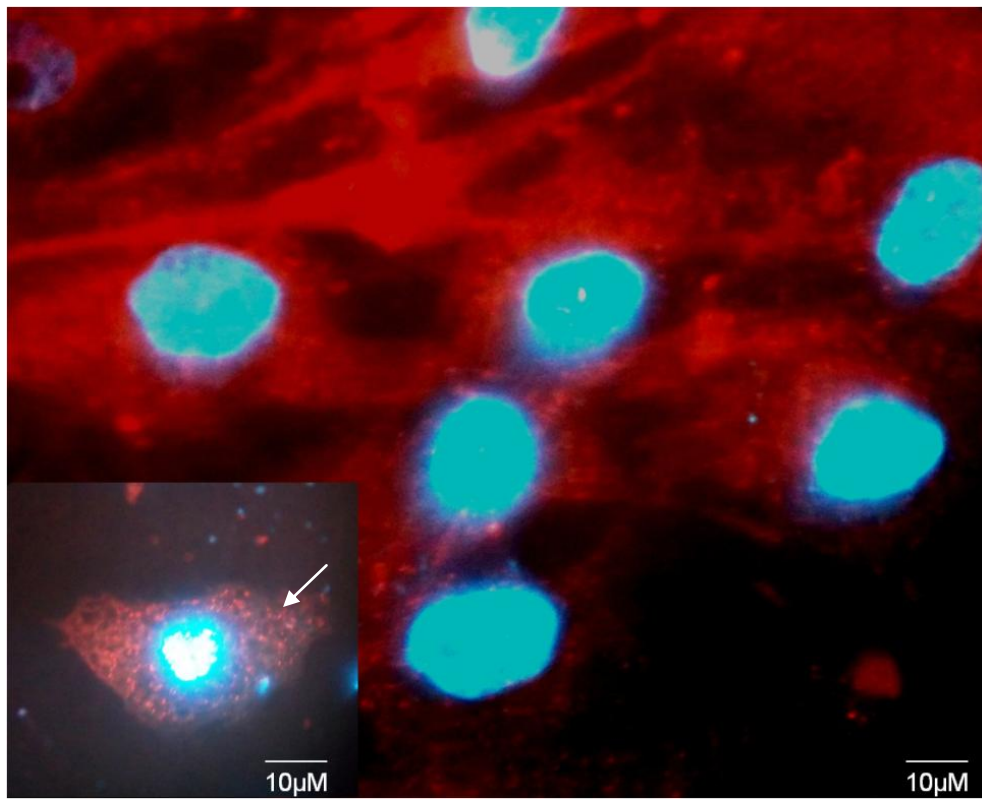


FIGURE 2

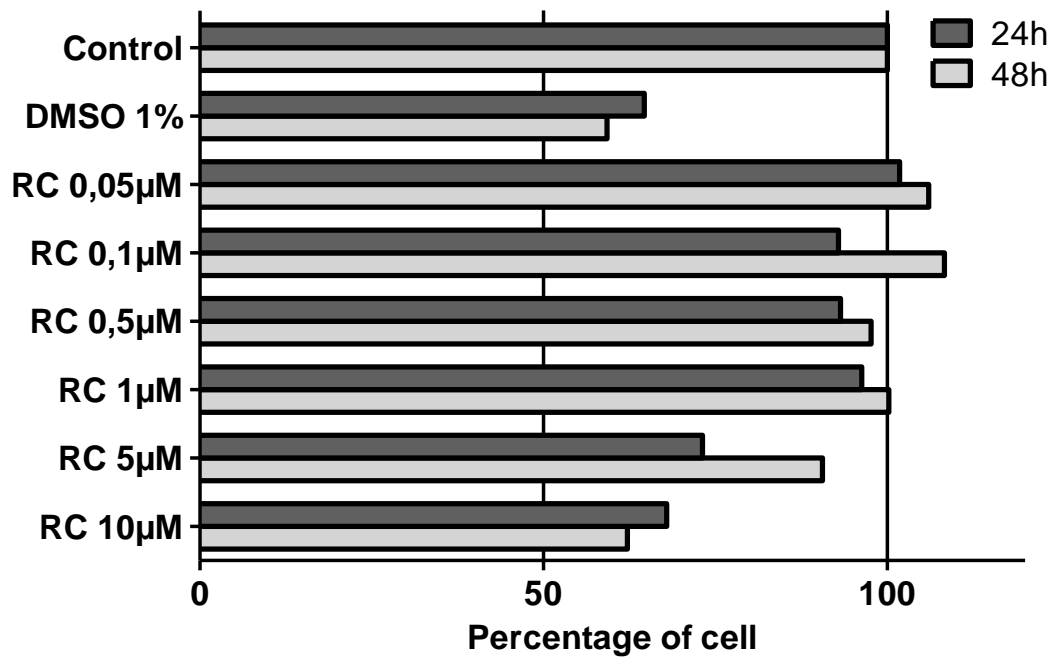


FIGURE 3

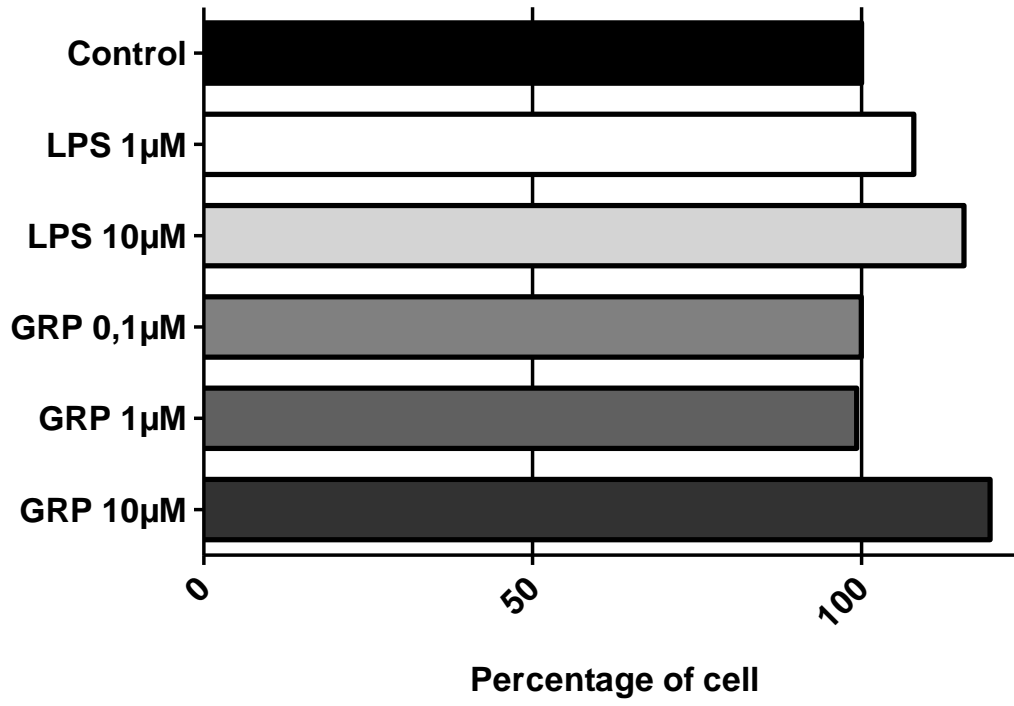
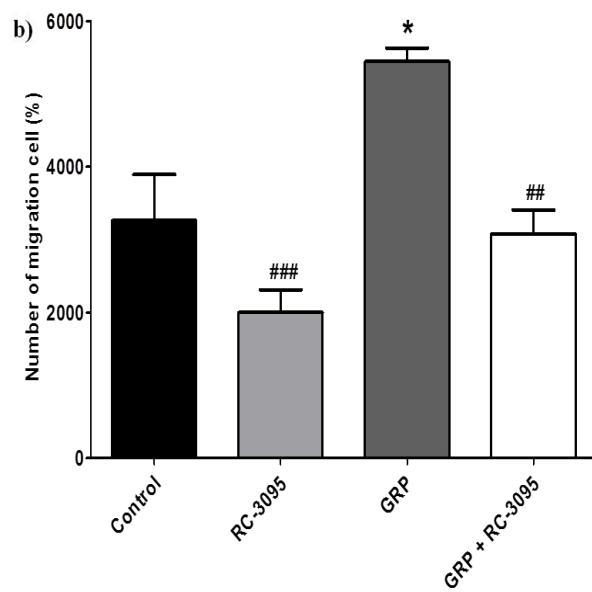
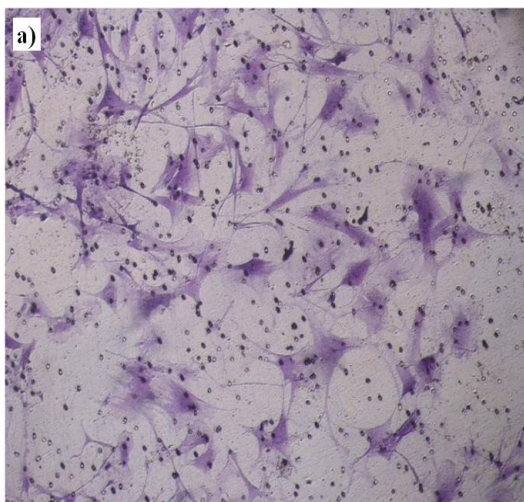


FIGURE 4



ANEXO

NORMAS DA REVISTA

Original research articles - Extended reports

These represent a substantial body of laboratory or clinical work. Extended reports should not exceed 3000 words; articles that exceed this word limit may be returned for revision before peer review. Additional data may be presented as supplementary information, which will be published online only should the article be accepted (this can be in any format: text, tables, images, videos, etc.). Extended reports should be presented in sections - namely:

1. **Abstract**

No more than 250 words, summarising the problem being considered, how the study was performed, the salient results and the principal conclusions under subheadings 'Objectives', 'Methods', 'Results', and 'Conclusions'.

2. **Key words**

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3. **Introduction**

Brief description of the background that led to the study (current results and conclusions should not be included).

4. **Methods**

Details relevant to the conduct of the study. Wherever possible give numbers of subjects studied (not percentages alone). Statistical methods should be clearly explained at the end of this section.

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6. **Discussion**

The nature and findings of the study are placed in context of other relevant published data. Caveats to the study should be discussed. Avoid undue extrapolation from the study topic.

7. **Acknowledgments and affiliations**

Individuals with direct involvement in the study but not included in authorship may be acknowledged. The source of financial support and industry affiliations of all those involved must be stated.

8. **References** (no limit - but usually below 50).

Please see References for further style guidance.

9. **Figure legends**

Maximum 6 tables and/or figures.

Please see Illustrations and tables for further style guidance.

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Journal article

13 Koziol-Mclain J, Brand D, Morgan D, et al. Measuring injury risk factors: question reliability in a statewide sample. *Inj Prev* 2000;**6**:148–50.

Chapter in book

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