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Title: Modified profile of matrix metalloproteinase-2 and -9 production by human Fallopian tube epithelial cells following infection *in vitro* with *Neisseria gonorrhoeae*

Authors: Paula I. Rodas [1], Doris Pérez [1,2], Claudia Jaffret [1,2], Yaquelin González [1], Carolina Carreño [1,3], Cecilia V. Tapia [4,5], Eduardo Osorio [6], Luis A. Velasquez [1], Myron Christodoulides [7]

Affiliations: [1] Center for Integrative Medicine and Innovative Science, Facultad de Medicina, Universidad Andres Bello, Santiago, Chile.

[2] School of Medical Technology, Faculty of Medicine, Universidad Andres Bello, Santiago, Chile.

[3] School of Medicine, Faculty of Medicine, Universidad Andres Bello, Santiago, Chile.

[4] Laboratorio Clínica Dávila, Santiago, Chile.

[5] Laboratorio de Micología Médica, Programa de Microbiología y Micología, ICBM, Facultad de Medicina, Universidad de Chile, Santiago, Chile.

[6] Servicio de Ginecología y Obstetricia, Clínica Dávila, Santiago, Chile

[7] *Neisseria* Research Group, Molecular Microbiology, Sir Henry Wellcome Laboratories, Clinical and Experimental Sciences, University of Southampton Faculty of Medicine, Southampton, England, SO16 6YD

Correspondence: Myron Christodoulides, *Neisseria* Research Group, Molecular Microbiology, Sir Henry Wellcome Laboratories, Clinical and Experimental Sciences, University of Southampton Faculty of Medicine, Southampton, England, SO16 6YD (m.christodoulides@soton.ac.uk)

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ABSTRACT

Epithelial shedding and scarring of Fallopian tube mucosa are the main consequences of sexually transmitted *Neisseria gonorrhoeae* infection and likely involves an imbalance of host extracellular matrix components (ECM) and their regulators such as matrix metalloproteinases (MMPs). In this brief report, primary human Fallopian tube epithelial cells were infected with *N. gonorrhoeae* and MMP patterns examined. Gonococcal infection induced a significant increase in secreted MMP-9 and an accumulation of cytoplasmic MMP-2 over time, but no significant MMP-3 or MMP-8 production was observed. Thus, MMP-9 in particular could play a role in tubal scarring in response to gonococcal infection.

Keywords: extracellular matrix; human Fallopian tube epithelial cells; matrix metalloproteinases; *Neisseria gonorrhoeae*

Running title: Gonococcal infection modifies MMP production by FTEC

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Abbreviations: extracellular matrix (ECM); human Fallopian tube (FT); lactate dehydrogenase (LDH); matrix metalloproteinase (MMP); multiplicity of infection (MOI); primary human Fallopian tube epithelial cells (FTEC); sexually-transmitted infection (STI)

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BACKGROUND

Neisseria gonorrhoeae (gonococcus) is the causative agent of the sexually-transmitted infection gonorrhoea, which continues to be a major public health problem because of the emergence of multidrug-resistant gonococci and the facilitation of HIV transmission (1). Gonorrhoea affects both men and women, but the most severe sequelae of untreated infection occur in the latter and include pelvic inflammatory disease, ectopic pregnancy and infertility as a consequence of ascending gonococcal infection that reaches the Fallopian tube (FT) epithelium (1). The tubal mucosa is colonized by gonococcal attachment to non-ciliated cells and the subsequent release of bacterial lipooligosaccharide (LOS) and peptidoglycan fragments by the gonococcus triggers the release of several pro-inflammatory cytokines such as IL-1α, IL-1β and TNF-α. In particular, release of TNF-α promotes direct damage to neighbouring ciliated cells, which leads to loss of ciliated activity, tissue sloughing and scarring as a mechanism of tissue repair (2,3).

Tissue repair involves the participation of the extracellular matrix (ECM), a protein complex network surrounding many tissues that plays a key role in numerous biological processes, including maintenance of reproductive function and tissue remodeling (4). Scar tissue is composed of ECM, but few studies have examined the roles of matrix metalloproteinases (MMPs) in scar formation and resolution. Composition of the ECM is regulated mainly by matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases that cleave most of the ECM proteins (5). Furthermore, pathogens such as *Chlamydia trachomatis* (6) and *Neisseria meningitidis* (7) have been reported to promote an imbalance of MMPs in order to model the ECM and thus gain access to host tissues (5). In the FT, Diaz *et al.* demonstrated that expression of genes encoding MMP-1, -2, -3 and MMP-7 through to MMP-16 was up-regulated during the

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periovulatory phase and generally reduced or down-regulated during the luteal phase, but the study did not report on MMP protein production (8). MMP expression and activation is subject to cytokine modulation (4) and epithelial cells in the genital tract are known to produce MMPs. However, the MMP expression patterns in FT epithelium infected by *N. gonorrhoeae* have not been studied. In this brief report, we tested the hypothesis that gonococcal infection of primary human FT epithelial cells *in vitro* as a model of the FT epithelium, modulates the production of a subset of important MMP proteins (MMP-2, -3, -8 and -9) as a potential mechanism that contributes to the excessive degradation of the ECM and tubal scarring.

MATERIALS AND METHODS

Strains: *Neisseria gonorrhoeae* P9 variant 17 (Pil$^+$ Opa$^+$) (P9-17) has been described previously (9) and was cultured on GC agar supplemented with IsoVitaleX (Becton Dickinson) at 37ºC with 5% (v/v) CO_2 for 18-24 hours. Colony phenotype was confirmed by stereomicroscopy.

Primary human Fallopian tube epithelial cells (FTEC) isolation and culture: Fallopian tube (FT) samples were obtained, after informed consent, from fertile donors undergoing hysterectomy for reasons unrelated to this study and without clinical history of sexually transmitted infections. The Ethics Board of Universidad Andres Bello and Clínica Dávila (Santiago, Chile) approved all protocols. The procedure for sample processing was previously reported (3,10). Briefly, samples were transported in cold, sterile PBS and then dissected and washed with TC199 medium. The tissue pieces (approximately 1 mm^3) were treated with 0.25% (v/v) trypsin (Hyclone) and incubated at 37ºC and 5% CO_2 (v/v) for 15 min. After adding of TC199 to stop trypsin reaction, digested tissue pieces were strained using a cell strainer (100 μm). The resulting cell suspension was centrifuged and the pellet was suspended in growth medium (TC-199 supplemented with 10% (v/v) decomplemented fetal bovine serum, 1 mM sodium pyruvate, 5 mg/ml human

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recombinant insulin) and antibiotics (50 IU/ml penicillin and 50 mg/ml streptomycin). Cells were seeded into cell culture flasks and incubated at 37ºC and 5% CO_2 (v/v) for 2 h to enable adherence of any fibroblasts, and then the medium containing non-adherent cells (epithelial cells) was centrifuged and the cells stored by cryopreservation until use. For infection assays, epithelial cells were grown at 37ºC with 5% (v/v) CO_2 on tissue culture flasks in growth medium for at least 5 days to obtain morphologically round 'island' colonies of epithelial cells. FTEC can also be characterized by immuno-histochemical staining with antibodies to specific cellular markers, as described previously (10). Then cell monolayers were digested with trypsin, centrifuged and washed three times with phosphate-buffered saline (PBS) pH7.4, and seeded onto 24-well tissue plates for at least 3 days to achieve 80-90% confluence. Using this procedure, any fibroblast contamination was detected morphologically by the presence of typical large, flat, elongated cells just 24 h post-seeding. After 5 days culture, a high confluence of non-ciliated FTECs (over 80%) was clearly differentiated by a 'cobblestone' appearance of epithelial cells and if fibroblasts were detected in any culture flasks, the cell cultures were discarded and not used for infection experiments.

Infection assays: FTEC monolayers (~ 3.0 x 10^6 cells/monolayer) were infected in duplicate with *N. gonorrhoeae* (MOI=10) for 8, 12 and 24 h in serum-free TC-199 medium. Supernatants were recovered, centrifuged (20 min at 13,000 g) and sterilized by filtration (0.2 µm filter). Lysates of FTEC were obtained after PBS washing by addition of medium to wells and disruption by mechanical lysis. Lysates were centrifuged and filtered as described for supernatants. All samples were stored at -80ºC until use. To determine FTEC viability during infection, release of lactate dehydrogenase (LDH) was measured into supernatants using the CytoTox® Non-Radioactive Cytotoxicity Assay (Promega), following the manufacturer's instructions.

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MMP enzyme-linked immunosorbent assay (ELISA): total intracellular and secreted (supernatant) contents of MMP-2, MMP-3, MMP-8 and MMP-9 in control and gonococcal infected FTEC monolayers were quantified using specific sandwich ELISA (R&D Systems), following the manufacturer's instructions.

Gelatin zymography: samples (7.5 µl) recovered from infected cells were loaded onto 12.5% (w/v) SDS-PAGE gels co-polymerized with 0.1% (w/v) gelatin (Sigma). Human, recombinant MMP-2 and MMP-9 (R&D Systems) were used as activity standard markers. After electrophoresis, gels were washed four times (15 min each wash) in 2.5% (v/v) Triton X-100 detergent and incubated with gentle agitation for 48h at 37ºC in 50 mM Tris-HCl buffer, pH7.5, containing 10 mM $CaCl_2$, 150 mM NaCl and 0.02% (w/v) sodium azide. Gels were rinsed gently three times with water, stained in 0.5% (w/v) Coomassie blue R-250 prepared in methanol:acetic acid:water (4:1:5) for 1h at room temperature with gentle agitation and de-stained in the same buffer without dye. Gelatin proteolysis was visualized as unstained bands on a blue background indicating gelatinase activity.

Statistical analysis: Data were provided by 8 independent experiments using FTEC from 8 different donors. Statistical analyses were performed using t–test to compare between control and infection samples taken at different time-points post-infection. P value <0.05 was considered significant.

RESULTS

In a prior study, expression of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) was constitutively expressed in human FT explants throughout the menstrual cycle, suggesting that

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their expression and activity was hormone-independent (8). However, in the current study, a modified pattern of MMP-2 protein expression was observed when FTEC were infected *in vitro* with live *N. gonorrhoeae*. A significant ($P<0.05$) time-dependent increase in intracellular MMP-2 levels was detected in lysates of FTEC infected with gonococci, compared with uninfected controls (Fig. 1A), with the latent pro-MMP-2 form of the zymogen more abundant than the active enzyme, as observed by gelatin zymography (Fig. 1A). In contrast, higher levels of the latent form of MMP-2 were observed in supernatants, but there were no significant differences in MMP-2 levels between infected and uninfected cell cultures ($P>0.05$) (Fig. 1B). In contrast to MMP-2, intracellular levels of MMP-9 were significantly reduced in lysates from FTEC cultures infected with live gonococci (Fig. 2A), with a predominant band identified by gelatin zymography as corresponding to the latent pro-MMP-9 zymogen. Maximal secretion of the pro-MMP-9 zymogen was observed by 24 h (Figure 2B). We have previously shown that gonococci adhere to and can subsequently invade FTEC (3,10), and in the current study, despite the change in morphological appearance over time from the typical epithelial ‘cobblestone appearance’ to a ‘rounded cuboid cell’ phenotype, FTEC viability was unaffected, as demonstrated by the lack of significant release of LDH (data not shown). In addition, during infection, MMP-3 and MMP-8 were not detected by ELISA.

DISCUSSION

Bacterial pathogens have the ability to modulate the host environment to achieve successful tissue colonization (5). In this brief report, we demonstrated that infection of primary FT epithelial cells *in vitro* did not induce MMP-3 or MMP-8 production, but modified the constitutive expression of MMP-2 and MMP-9. Expression of MMP-1 mRNA and protein has been detected in human FT (11) and we have previously observed gelatinolysis in FT samples

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that may have been directed by MMP-1 (unpublished observations). Several studies have reported that MMP expression is induced during bacterial infection; in particular *Chlamydia trachomatis,* which similarly targets FTEC, promoted a differential expression of MMP-2 and MMP-9 (6,12). Interestingly, both MMPs have been linked to oviduct fibrosis and *Chlamydia*-induced scarring (6,13), which are also the main sequelae generated by gonococcal salpingitis. However, gonococci and *Chlaymdia* appear to display different modification profiles for MMP expression in FTECs. *N. gonorrhoeae* infection appeared to promote high release of pro-MMP-9 zymogen by 24h and the accumulation of intracellular MMP-2 but with no significant increase in extracellular pro-enzyme, whereas *C. trachomatis* infection tended towards inducing MMP-2 secretion instead of MMP-9 by human FTEC (6) and no data were presented for intracellular MMPs. Interestingly, our data are similar to the observations from the *Chlamydia muridarum* model of mouse urogenital tract infection, in which MMP-2 expression was reported also to be constitutive and unchanged during infection, whereas the levels of MMP-9 were found to be significantly increased in homogenized murine upper reproductive tract tissue (uterine and oviduct) (13). Furthermore, expression and activity of MMP-9 was significantly up-regulated in the inflamed conjunctiva of humans infected with *C. trachomatis* (14). However, the molecular events involved in MMP modulation by these pathogens, particularly the participation of bacterial protein effectors and/or virulence factors, remains to be determined.

In conclusion, our brief report shows that gonococcal infection appeared to modify the pattern of MMP-2 and MMP-9 protein expression in FTECs *in vitro*. The roles of MMP-2 and MMP-9 in tissue remodeling that involves cell migration and laying down new epithelium are not clear. Like other MMPS, MMP-2 and MMP-9 selectively degrade many components of the ECM (including gelatin, collagens IV and V, elastin, fibronectin and vitronectin) but also can process growth factors (latent TGF-β) and cytokines (pro IL-1β and IL-1β, latent TNF-α) that reside in

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the ECM, which are involved in inflammation, tissue growth and repair. Interestingly, the plasminogen/plasmin system widely involved in the fibrinolysis pathway is epistatic to MMP-9 production (15). Thus, if MMP-9 production in particular can lead to aberrant ECM degradation and an inflammatory response, we hypothesize that this event is likely to be beneficial to *N. gonorrhoeae* for successful colonization and dissemination throughout the reproductive tract. Further studies are required to test this hypothesis and to identify the nature of the gonococcal molecules that mediate interactions with host ECM during infection, which may represent new pharmacological targets for intervention in gonorrhoea.

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FOOTNOTE: FINANCIAL SUPPORT.

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FOOTNOTE: CONFLICT OF INTEREST.

The authors declare no competing financial interests or other relationships relevant to the study. This work was presented at the XIXth International Pathogenic Neisseria Conference, Asheville (NC), USA (2014).

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FIGURE LEGENDS

Fig. 1. Measurement of MMP-2 protein in FTEC infected with *Neisseria gonorrhoeae*. FTEC monolayers were infected with *N. gonorrhoeae* P9-17 (MOI=10) for 8, 12 and 24 h. Intracellular content was quantified from FTEC cell lysates (A) and secreted MMP-2 from the supernatants (B) by ELISA and gelatin zymography. C, control (uninfected cells); I, infection (*N. gonorrhoeae* infected cells). (+), positive control of MMP-2 protein. ELISA data are represented as the means and the standard error of the mean (SEM) of n=8 experiments with 8 independent FTEC donors. The zymograph is representative of the n=8 infection experiments.*$p<0.05$.

Fig. 2. Measurement of MMP-9 FTEC infected with *Neisseria gonorrhoeae*. FTEC monolayers were infected with *N. gonorrhoeae* P9-17 (MOI=10) for 8, 12 and 24 h. Intracellular content was quantified from FTEC cell lysates (A) and secreted MMP-9 from the supernatants (B) by ELISA and gelatin zymography. C, control (uninfected cells); I, infection (*N. gonorrhoeae* infected cells). (+), positive control of MMP-9 protein. ELISA data are represented as the means and the standard error of the mean (SEM) of n=8 experiments with 8 independent FTEC donors. The zymograph is representative of the n=8 infection experiments.*$p<0.05$.

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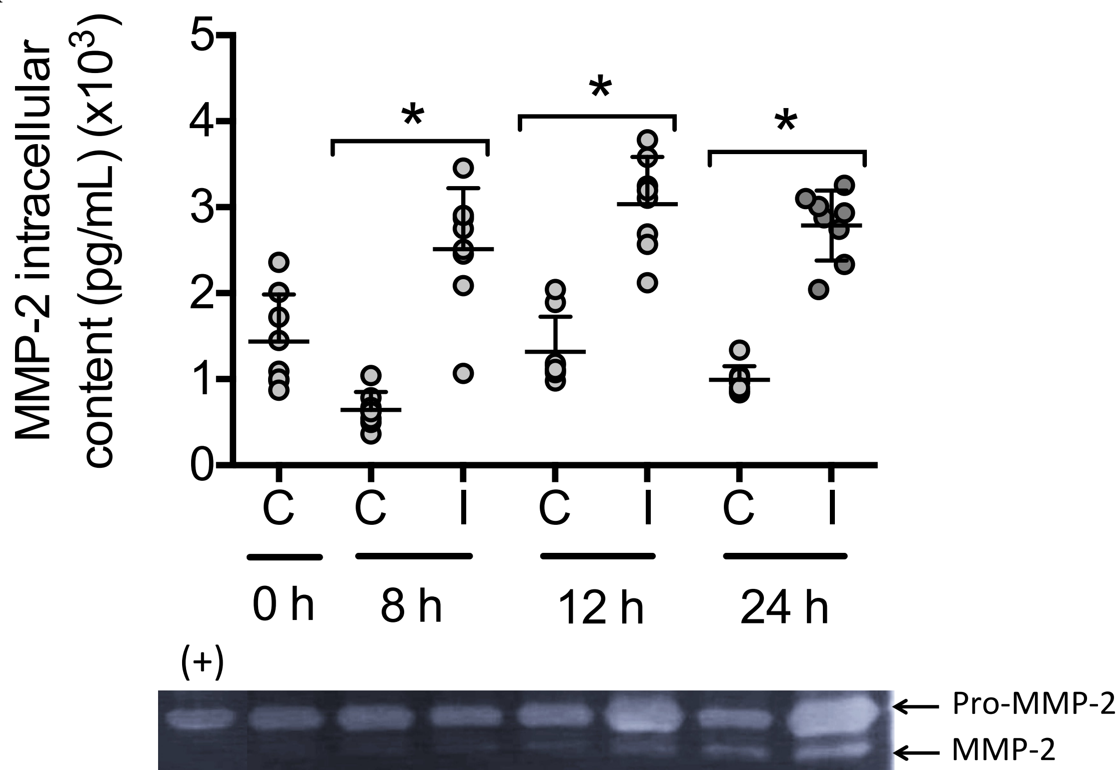
Figure 1

A



B

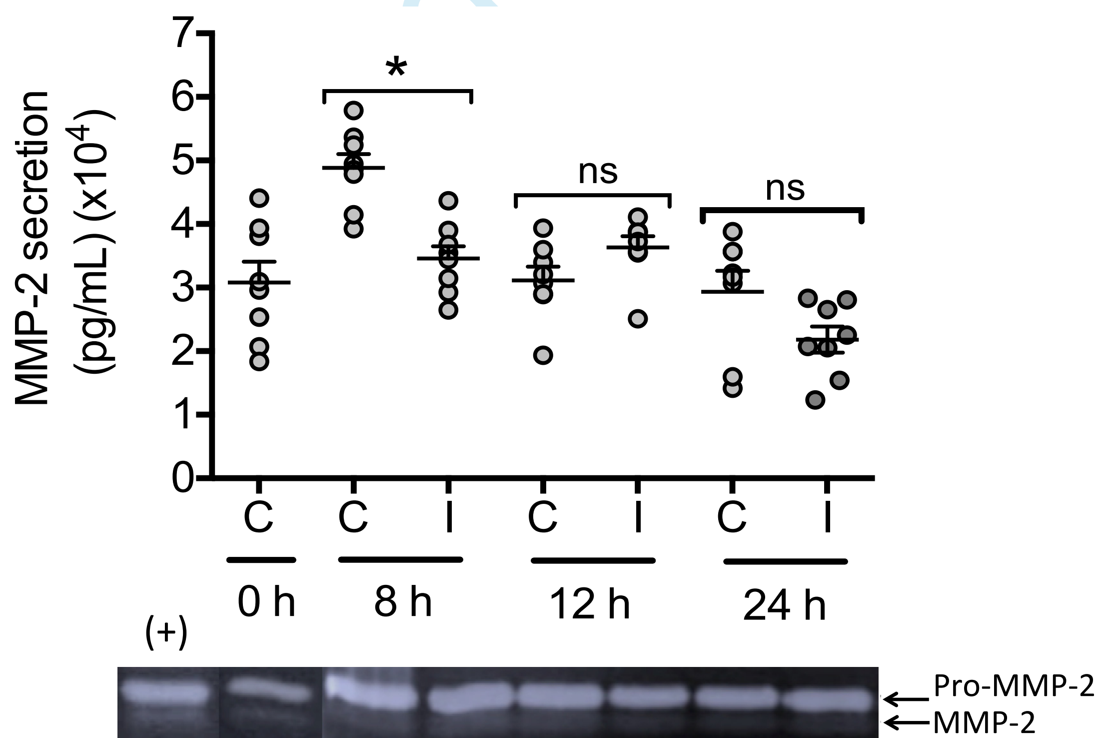


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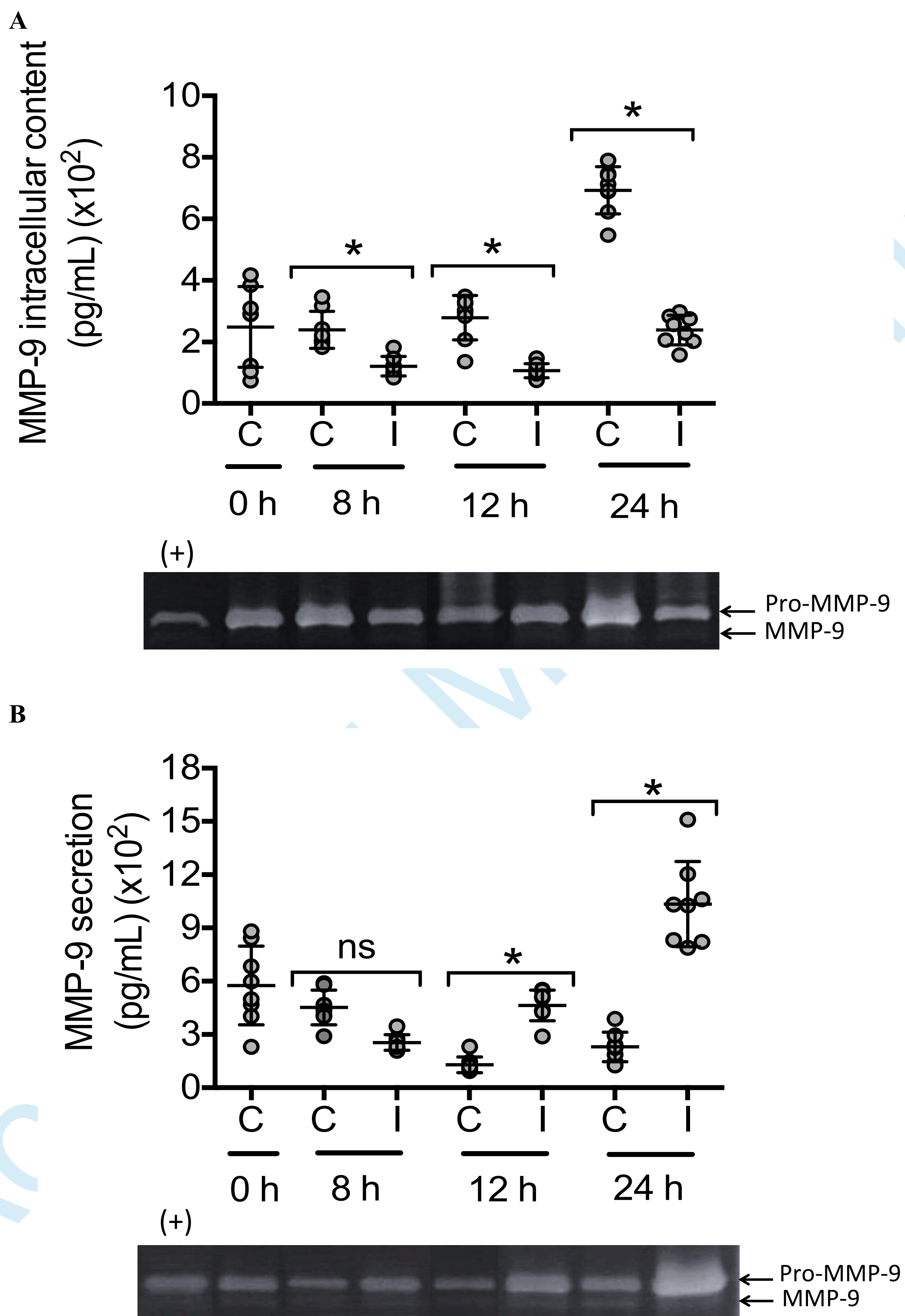
Figure 2



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