Antimicrobial and anti-inflammatory activity of Cystatin C on human gingival fibroblast incubated with *Porphyromonas gingivalis*

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Background. Periodontal disease is considered one of the most prevalent chronic infectious diseases, often leading to the disruption of tooth-supporting tissues, including alveolar bone, causing tooth mobility and loss. *Porphyromonas gingivalis* is considered the major etiological agent of this disease, having a plethora of virulence factors, including, lipopolysaccharides (LPS), hemolysins, and proteinases. Antimicrobial peptides are one of the main components of the innate immune response that inhibit the growth *of P. gingivalis***.** The aim of this study was to analyze the antimicrobial activity of cystatin C and to assess the effect on the inflammatory and anti-inflammatory cytokines, the production of reactive oxygen species, and in the release of nitric oxide by human gingival fibroblasts incubated with *P. gingivalis* in the presence and absence of cystatin C. **Methods.** *P. gingivalis* ATCC 33277 was exposed to cystatin C for 24h and co-cultured with human gingival fibroblasts (HGFs) ATCC CRL-2014. The effect of cystatin on growth of *P. gingivalis* and HGFs was evaluated. Pro-inflammatory (TNFα, IL-1β) and anti-inflammatory (IL-10) cytokines were determined by ELISA in the supernatants of HGFs incubated with *P. gingivalis* exposed to cystatin C. Additionally, nitrites and reactive oxygen species (ROS) production were evaluated. **Results.** Cystatin C inhibited the growth of *P. gingivalis* without affecting HGFs. Incubation of HGFs with *P. gingivalis* led to a significant increase of TNF-α and IL-1β. In contrast, HGFs incubated with *P. gingivalis* exposed to cystatin C showed a decreased production of both cytokines, whereas IL-10 was enhanced. Incubation of HGFs with *P. gingivalis* led to an increase of nitric oxide (NO) and ROS production, which was reduced in the presence of the peptide. **Conclusions .** Cystatin C inhibits the growth of P. gingivalis and decreases the inflammatory cytokines, ROS, and NO

production during infection of HGFs with *P. gingivalis* . Knowledge on the antimicrobial and immunomodulatory properties of cystatin C could aid in the design of new therapeutic approaches to facilitate the elimination of this bacterium to improve the treatment of periodontal disease.

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23 **Abstract**

25 **Background.** Periodontal disease is considered one of the most prevalent chronic infectious

26 diseases, often leading to the disruption of tooth-supporting tissues, including alveolar bone,

27 causing tooth mobility and loss. *Porphyromonas gingivalis* is considered the major etiological

28 agent of this disease, having a plethora of virulence factors, including, lipopolysaccharides,

29 hemolysins, and proteinases. Antimicrobial peptides are one of the main components of the

30 innate immune response that inhibit the growth *of P. gingivalis***.** The aim of this study was to

31 analyze the effect of Cystatin C effect on the inflammatory and anti-inflammatory cytokines, the

32 production of reactive oxygen species, and in the release of nitric oxide by human gingival

33 fibroblasts incubated with *P. gingivalis* in the presence and absence of cystatin C.

34 **Methods.** *P. gingivalis* ATCC 33277 was exposed to cystatin C for 24h and co-cultured with

35 human gingival fibroblasts (HGFs) ATCC CRL-2014. The effect of cystatin on growth of *P.*

36 *gingivalis* and HGFs was evaluated. Pro-inflammatory (TNFα, IL-1β) and anti-inflammatory

37 (IL-10) cytokines were determined by ELISA in the supernatants of HGFs incubated with *P.*

38 *gingivalis* exposed to cystatin C. Additionally, nitrites and reactive oxygen species (ROS)

39 production were evaluated.

40 **Results.** Cystatin C inhibited the growth of *P. gingivalis* without affecting HGFs. Incubation of

41 HGFs with *P. gingivalis* led to a significant increase of TNF-α and IL-1β. In contrast, HGFs

42 incubated with *P. gingivalis* exposed to cystatin C showed a decreased production of both

43 cytokines, whereas IL-10 was enhanced. Incubation of HGFs with *P. gingivalis* led to an

44 increased NO and ROS production, which was reduced in the presence of the peptide.

45 **Conclusions.** Cystatin C inhibits the growth *of Porphyromonas gingivalis* and decreases the

46 inflammatory cytokines, ROS, and NO production during infection of HGFs with *P. gingivalis.*

47 Knowledge on the antimicrobial and immunomodulatory properties of cystatin C could aid in the

48 design of new therapeutic approaches to facilitate the elimination of this bacterium to improve

49 the treatment of periodontal disease.

50

51 **Introduction**

52

53 Periodontitis is a chronic infectious disease, characterized by an exacerbated inflammatory

54 response and progressive loss of tooth supporting tissues (Könönen et al., 2019) *Porphyromonas*

55 *gingivalis* is a periodontopathogen bacterium implicated as a major, etiological agent in

56 periodontitis (van Winkelhoff et al., 2002). This bacterium has been recovered from periodontal

57 pockets in a high percentage (75.8%) of patients with periodontitis (Rafiei et al., 2017)

58 The most abundant cell types in periodontal connective tissues are gingival fibroblasts

59 (GF),where they participate in the repair of periodontal tissues during inflammatory periodontal

60 diseases (Lee, et al., 2012). GF also promotes periodontal wound healing (Smith et al., 2019;

61 Baek et al., 2013)

62 Furthermore, LPS of *Porphyromonas gingivalis* increases their superoxide concentrations after

63 the exposure to human gingival fibroblasts (HGFs) (Staudte et al., 2010; Gölz et al., 2014).

64 Thus, these cells can also participate in the progression of periodontitis, inducing the release of

65 inflammatory such as mediators nitric oxide cytokines,and reactive oxygen species (ROS), and

66 nitric oxide(How et al., 2016; Kirkwood et al., 2007; Gölz et al., 2014; Herath et al., 2016).

67   Cytokines are involved in the initiation and progression of periodontal disease (Ramadan et

68 al., 2020) Even though secreted cytokines promote the elimination of bacteria, the

69 overproduction of pro-inflammatory cytokines may participate directly in periodontal

70 breakdowns, such as the breakdown of collagen periodontal attachment loss, and alveolar bone

71 resorption (Gabay, Lamachia & Palmer, 2010). TNF-α and IL-1β are the major secreted pro-

72 inflammatory cytokines, that are important markers of periodontitis progression and severity. and

73 they are also the main inducers of effector molecules that cause the breakdown of periodontal

74 tissues (Gomes et al., 2016). TNF-α and IL-1β are produced by several cell types including

75 dendritic cells, macrophages, periodontal ligament cells, osteoblasts, and gingival fibroblasts and

76 can act as multifunctional molecules (Cheng et al., 2020). IL-1β promotes production of

77 metalloproteinases (MMPs), which are involved in the extracellular matrix degradation and, in

78 turn, bone resorption and periodontal tissue destruction (Aleksandrowicz et al., 2021). TNF-α,

79 participates in the bone resorption process, inducing RANK expression in osteoclast precursors

80 and RANKL expression in osteoblast (Pan, Wang & Chen, 2019). In addition, TNF-α and IL-1β

81 also induce reactive oxygen species (ROS) generation in periodontal tissue (Wang et al., 2014),

82 where oxidative stress has been shown to be involved in periodontitis (Tomofuji et al., 2006;

83 Maruyama et al., 2011).These pro-inflammatory mediators are required for the immune defense

84 against bacteria, yet their uncontrolled activity leads the accumulation of ROS (superoxide

85 radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen) (Gölz et al., 2014). Even

86 though these products stimulate proliferation and differentiation of cultured human periodontal

87 ligament fibroblasts at low concentrations, their presence in higher concentrations can induce

88 pathogen killing and cytotoxic effects on periodontal tissues and pathogen killing (Chapple &

89 Matthews 2007). Zhu et al. (2020) demonstrated that after the stimulation of HGFs with LPS*,*

90 ROS production in mitochondria (mtROS) was significantly enhanced,,these results indicate that

91 oxidative stress can be induced during periodontitis (Liu et al., 2021). It is noteworthy that *P.*

92 *gingivalis* is resistant to oxidative burst killing due to its antioxidant enzymes, such as thiol, and

93   rubrerythrin. Furthermore, these bacteria accumulate a hemin layer on the cell surface that

94   protects the bacteria from oxidative stress (Wang et al., 2014; Henry, McKenzie, Robles &

95   Fletcher, 2012).

96   On the other hand**,** IL-10, an anti-inflammatory cytokine that suppresses the inflammatory

97   responses (Al-Rasheed et al., 2003), also protects from tissue destruction by inhibiting both

98   matrix metalloproteinases (MMPs) and receptor activators for nuclear factor-kB (RANK)

99   systems, leading to the differentiation and activation of osteoclasts (Garlet et al., 2006).

100   Stimulation with bacteria or bacterial components like LPS induce the production of

101   inflammatory cytokines, such as interleukin 1, -6, -8, and nitric oxide (NO), in human

102   monocytes, endothelial cells, macrophages, and gingival fibroblasts (Gutierrez-Venegas et al.,

103   2005; Staudte et al., 2010; Gölz et al, 2014). *P. gingivalis* triggers the production of NO by

104   activating the expression of inducible nitric oxide synthases (Sun, et al. 2010; Brennan, Thomas,

105   & Langdon, 2003). It is noteworthy that it can resist NO stress and maintain nontoxic

106   intracellular NO concentrations (Zumf, 2002). Thus, a high concentration of NO fails to

107   eliminate this bacterium, yet it can exert a deleterious effect on the periodontal tissue, favoring

108   vasodilation and diminishing platelet aggregation, which contributes to gingival bleeding. These

109   toxic effects on the surrounding tissue increase the severity of periodontitis (Boutrin et al., 2012).

110   It has been suggested that the inducible nitric oxide syntase (iNOS) may be involved in

111   periodontal pathogenesis (Batista et al., 2002), since common periodontal pathogens can induce

112   the expression of iNOS in various host cells, including HGFs (Sosroseno, et al., 2009).

113   Additionally, cytokines and chemokines expressed by gingival fibroblasts in response to *P.*

114   *gingivalis* can accumulate and their subsequent action on leukocytes is modulated due to the

115   enzymatic activity of *P. gingivalis*-derived proteinases, that cleave and inhibit their biological

116   properties (Calkins et al. 1998; Kobayashi, Isogi & Hirose 2003**;** Palm, Khalaf & Bengtsson,

117   2015). The production of *P. gingivalis* cysteine proteinases are associated with the growth and

118   establishment of *P. gingivalis,* they .are divided into arginine-specific (Rgp) and lysine-specific

119   (Kgp) proteinases .Additionally, these cysteine proteases exert potent immunomodulatory effects

120   on human gingival fibroblasts. The main causative factor of tissue damage involved in the

121   disease progression, could be the gingipains of the bacterium ,even though *P. gingivalis* is

122   considered an opportunistic pathogen. Thus, control of proteolytic enzymes of *P. gingivalis*

123   could represent an interesting target for the treatment of periodontitis (Torbjörn, Atika &Hazem,
124   2015).
125   Antimicrobial peptides (AMPs) are part of the innate defense system in the oral cavity, where
126   cystatins play an important role. Cystatin C belongs to the type 2 family of the cystatin
127   superfamily, it is ubiquitously distributed in plants and animals (Shamsi & Bano, 2017). In the
128   parotid gland of humans, it is present in saliva at a concentration of 0.9 μg/mL (Gorr, 2012).
129   The main function of cystatin C is the inhibition of cysteine proteases by binding to their active
130   sites (Palm, Khalaf & Bengtsson, 2015). It also exerts several immunomodulatory functions and
131   possesses the ability to regulate innate immune responses (Vray, Hartmann & Hoebeke, 2002)
132   The aim of this study was to assess the effect that cystatin C exerts on cytokine production, NO
133   and ROS production by human gingival fibroblasts incubated with *P. gingivalis* in order to be
134   able to evaluate its potential therapeutic use against one of the main etiological agent causing
135   periodontitis, as well as its potential impact on the severity of periodontal disease.
136
137   **Materials & Methods**
138
139   **Cells culture**
140   Human gingival fibroblasts (HGFs) (ATCC, CRL-2104) were seeded at a density of 5×10^3
141   cells per cm^2 and cultured in 75 cm^2 culture flasks in a water
142   saturated atmosphere *at* 37°C and 5% CO_2 and maintained in Dulbecco´s modified Eagle high
143   glucose medium (Sigma Aldrich, Saint Louis, MO, USA), supplemented with 10% fetal bovine
144   serum (GIBCO BRL, Gaithersburg, MD, USA), containing 10 U penicillin / 25 μg streptomycin
145   /mL) (Sigma Aldrich). The fibroblasts were cultured to confluence, at a density of 2.5×10^5
146   cells/mL, washed twice with phosphate-buffered saline, and dissociated with 0.25% trypsin and
147   1 mM EDTA for 5 min at 37°C, 5% CO_2 (Sigma Aldrich, Saint Louis, MO, USA). The cells
148   were used at passages 3-7.
149
150   **Bacterial growth**
151
152   *P. gingivalis* strain ATCC 33277 was cultured in brain-heart-infusion and in broth-heart-brain
153   extract (BHI; BD Bioxon, Milan, Italy) containing 5 μg/mL of hemin (Sigma-Aldrich, Munich,
154   Germany) and 1 μg/mL of menadione (Sigma-Aldrich) under anaerobiosis using the anaerobic
155   BBL-GasPak jar system. (BD Biosciences) at 37°C for 24 h

156 After 24 h of culturing, bacteria were harvested by centrifugation for 10 min at 10000 rpm

157 and then washed and resuspended in Krebs-Ringer-Glucose (KRG) buffer (120 mM NaCl, 4.9

158 mM KCl, 1.2 mM MgSO$_4$, 1.7 mM KH$_2$PO$_4$, 8.3 mM Na$_2$HPO$_4$, 10 mM glucose, and 1.1 mM

159 CaCl$_2$, pH 7.3). Bacterial growth was monitored spectrophotometrically (Jenway Genova R0027,

160 Fischer Scientific, USA) at 675 nm. The bacterial density was visually adjusted to a turbidity of

161 0.5 McFarland (1 × 10^8 colony-forming units; (CFU/mL) (Mc Farland, 1907 Emani et al., 2014).

162 Ethical approval was given by the Ethics Committee of the School of Medicine (UNAM) with

163 reference number C54-11.

164

165 **Antibacterial assay**

166 Lyophilized Cystatin C was obtained from *Pichia Pastoris* (Sigma Aldrich, St. Louis, MO)

167 and reconstituted in Tris Base NaCl Buffer (pH 7.4). Minimum inhibitory concentrations (MIC)

168 of Cystatin C was determined using the microdilution method in 96-well microtiter plates

169 (Costar, Corning Life Sciences) (Eloff, 1998; Jadaun et al., 2007). Briefly, an inoculum of *P.*

170 *gingivalis* (1 x 10^6 CFU/ mL) containing KRG Buffer was placed in each well. Subsequently,

171 different cystatin C concentrations (0.1, 0.3, 0.5, 0.7, 0.9 µg/mL) were incubated with the

172 bacteria, for 1, 12, 24, and 48 h, under anaerobiosis conditions, at 37°C. After the incubation

173 period, 20 µL of Presto Blue Cell Viability Reagent (Invitrogen, Thermo Fisher Scientific) per

174 well were added. The plates were incubated for 30 min at 37°C in the dark. Finally, the plates

175 were read in a microplate reader (Multiskan SkyHigh Microplate Spectrophotometer), at a 675

176 nm wavelength.

177

178 **Cell Viability assay**

179 HGFs were seeded at a density of 1x10^5 cells/well in 24-well plates for 24 h, at 37°C with 5%

180 CO2. Different concentrations of Cystatin C (0.1, 0.3, 0.5,0.7,0.9 µg/ml) were added and

181 incubated for 24 h. After incubation time, 25 µl of XTT/PBS solution (4 mg/4ml) were added per

182 well, for 40 minutes at room temperature, in the dark. Subsequently, microplate plates were read

183 at a wavelength of 450 nm in a microplate spectrophotometer (Multiskan SkyHigh Microplate

184 Spectrophotometer).

185

186 **Treatment of human gingival fibroblasts (HGFs) with *P. gingivalis***

187 | Human gingival fibroblasts, at a seeding density of 5×10^5/well, were cultured in a Costar®

188 24-well plate (Corning Life Sciences, Corning, NY, USA) in D-MEM medium at 37°C in an

189 atmosphere of 5% CO_2. After the incubation period, fresh medium without antibiotics was added

190 to HGFs, before they were treated with *P. gingivalis*. HGFs were stimulated with bacteria, at

191 multiplicities of infection (MOI) of 1:100 for 24 h, and with cystatin C at a concentration of 0.3

192 μg/mL at 37°C for 24 h, to perform cytokine assays, and evaluate ROS, and NO. Control groups

193 include HGFs without stimulation or stimulated with LPS and peptidoglycans.

194

195 **Cytokine assays**

196   For cytokine assays, HGFs were incubated with *P. gingivalis* (MOI 1:100) and /or cystatin C

197 at a concentration of 0.3 μg/mL at 37°C for 24 h. Control groups included HGFs without

198 stimulation or stimulated with LPS 100 ng/mL (LPS from *Escherichia coli* O111:B4, Sigma

199 Aldrich), or with peptidoglycan 10μg/mL (Peptidoglycan from *Staphylococcus aureus*, Sigma

200 Aldrich). ELISAs were performed to determine TNF-α, IL-1β, and IL-10, using the Ready-Set-

201 Go! ELISA kits (BD Biosciences, Cytokine ELISA Protocol, San Diego, CA, USA), according

202 to the manufacturer's protocol. Dilutions were prepared in dilution buffer. Briefly, 96-well flat-

203 bottom plates (Costar®, Corning Life Sciences) were coated with anti-human TNF-α, IL-1β, or

204 IL-10 monoclonal antibodies (BD Biosciences, Pharmingen). After blocking with the assay

205 solution (PBS-0.5% casein diluted in 1 M NaOH) overnight at 4°C to avoid non-specific

206 binding, 100 μL of standard TNF-α, IL-1β, or IL-10 (BD Bioscience, Pharmingen) of

207 supernatants were added. The microplate was washed to remove unbound enzyme-labeled

208 antibodies. The amount of horseradish peroxidase bound to each well was determined by the

209 addition of a substrate solution. The reaction was stopped by the addition of sulfuric acid and the

210 plates were read at 405 nm (ELISA microplate reader, Bio-Rad, Hercules, CA, USA).

211   The cytokine concentration was calculated by regression analysis from a standard curve. The

212 detection limit of the assay was 15 to 2000 pg/mL.

213

214   **Measurement of NO production**

215   The NO production by HGFs incubated with *P. gingivalis* and/or cystatin C at 37°C was

216 assayed by measuring the accumulation of nitrate in culture supernatants. Briefly, HGFs were

217 stimulated with *P. gingivalis* (MOI 1:100) and with 0.3 μg of cystatin C, at 37°C for 24 h.

218   Thereafter, 100 μL of Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine

219   dihydrochloride, and 2.5% phosphoric acid) (Sigma Aldrich) were added at equal volumes of

220   culture supernatants in a 96- well plate (Costar®, Corning Life Sciences) and left at room

221   temperature for 30 min. The absorbance of these supernatants was read at 550 nm (Multiskan

222   SkyHigh Microplate Spectrophotometer) and the nitrate concentrations were calculated from a

223   standard curve established with serial dilutions of $NaNO_2$ (Sigma-Aldrich) in the culture

224   medium. Control groups included HGFs without stimulation or stimulated with LPS or

225   peptidoglycan.

226

227   **Detection of Reactive Oxygen Species (ROS)**

228   HGFs were seeded on 24-well plates (Costar®, Corning Life Sciences) at a density of (5 x

229   10^5), infected with *P. gingivalis* (MOI 1:100) and stimulated with 0.3 μg/ml of cystatin C at

230   37°C for 24 h. The cells were incubated with 100 μg/ ml of 2,7

231   dichlorodidrofluoroescein diacetate (H2-DCFDA) [2 μM/mL] for 30 min in the dark at room

232   temperature. Cells were rinsed twice with PBS, pH 7.2 and detached from the wells with 0.25%

233   Trypsin/EDTA (Sigma Aldrich). The samples were resuspended in PBS, pH 7.2, with 1% FBS

234   and analyzed on a FACS Canto II BD Biosciences flow cytometer. Data analysis was performed

235   using FlowJo software (USA). Control groups included HGFs without stimulation or stimulated

236   with LPS or peptidoglycan.

237

238   **Statistical analysis**

239   Experimental and control conditions were statistically compared for significance using analysis

240   of variance (ANOVA), followed by Benferroni correction. The predetermined level of

241   significance was $p < 0.05$. Statistical analysis was performed with the GraphPad, Prism v.6

242   software (GraphPad Software, Inc., CA, USA).

243

244   **Results**

245   **Effects of cystatin C on growth of *P. gingivalis* and viability of HGFs**

246   The antimicrobial activity of cystatin C on *P.gingivalis* was analyzed in a time and dose-

247   dependent manner as shown in (Fig. 1a). It reached its maximal antimicrobial activity at 24 h

248   with concentrations between 0.1 and 0.3 μg/mL.

249   The concentration of 0.3 µg/mL inhibited 75% of bacteria growth after 24h of incubation

250   when compared to the control group (p <0.05). Inhibition of bacterial growth (83.3%) was

251   observed after 48 h of culture (p <0.05). At a concentration of 0.9 µg/mL a marked growth

252   inhibition was observed throughout the incubation time. All the analyzed concentrations of

253   cystatin C showed no effect on the viability of HGFs cell, as illustrated in (Fig. 1b). These

254   findings reveal the antimicrobial activity of cystatin C against *P. gingivalis* and did not affect the

255   viability of HGFs. Hence, we decided to perform all the experimental assays with a cystatin C

256   MIC at 0.3 µg/mL.

257

258   **Effect of cystatin C on the production of pro- and anti-inflammatory cytokines**

259

260   TNF-α and IL-1β were evaluated in supernatants of HGFs incubated with *P. gingivalis* and

261   cystatin C (0.3 µg/mL) for 24 h. *P. gingivalis* induced the production of 1000 pg/ mL and 750

262   pg/mL of TNF-α and IL-1β, respectively, when compared to the control group (p = 0.0001) (Figs

263   2a, 2b). However, when HGFs were incubated with the bacteria and cystatin C, a statistically

264   significant decrease was observed in the TNF-α (p = 0.0001) and IL-1β (p < 0.05) productions,

265   compared to HGFs. In contrast, no changes were observed in IL-10 production by HGFs

266   incubated with *P. gingivalis* alone, when compared to controls, whereas cystatin C stimulated de

267   production and secretion of IL-10 (500 pg/mL). Furthermore, the co-incubation of *P. gingivalis*

268   with cystatin C significantly increased the production of IL-10 (900 pg/mL), when compared

269   with the control group and with HGFs infected with the bacterium (p = 0.0001), (Fig.2c). These

270   results suggest that cystatin C participates in the regulatory inflammatory process, by reducing

271   inflammatory cytokines and increasing anti-inflammatory cytokines.

272

273   **Cystatin C decreases ROS and NO production on HGFs incubated with *P. gingivalis***

274   A significant increase was observed in the production of ROS and NO in HGFs incubated

275   with *P. gingivalis*, compared to the controls (p = 0.0001). No significant differences were

276   observed in the production of ROS in HGFs incubated with cystatin C (p >0.05) (Fig.3a). In

277   contrast, a significant decrease in ROS was observed after the incubation of HGFs with *P.*

278   *gingivalis* and cystatin C, compared to the control (p = 0.001), (Fig. 3a).

279   Furthermore, a significant increase of NO (9 µM) was observed after the incubation of HGFs

280   with *P. gingivalis,* when compared with the control group (p = 0.0001). Yet when HGFs were

281 incubated with *P. gingivalis* and cystatin C, a decrease of NO (3 µM) (p = 0.001) was observed

282 with regard to the incubation with *P. gingivalis* alone (Fig. 3b).

283

284 **Discussion**

285   In this study, we analyzed the antimicrobial activity of cystatin C against *P gingivalis*, which

286 contributes to the development of chronic periodontitis. The immunological responses occurring

287 in HGFs after the infection with this key periodontal pathogen were evaluated. *P. gingivalis*

288 exhibits a variety of virulence factors that enable it to colonize oral soft tissues and evade

289 immune responses. It has been demonstrated that *P. gingivalis* triggers and suppresses the

290 immune responses in HGFs, suggesting that the pathogenic effects of *P. gingivalis* are mainly

291 related to the action of gingipains, which participate in the inflammatory and immune response

292 of HGFs (Palm, Khalaf & Bengtsson, 2015; Bengtsson, Khalaf & Palm, 2015). Additionally, *P.*

293 *gingivalis* has a direct modulatory function on the immune response of fibroblasts through the

294 catalytic activities of gingipains, targeting fibroblast-derived inflammatory mediators at the

295 protein level (Palm, Khalaf & Bengtsson, 2013). *P. gingivalis* secretes three related cysteine

296 proteases (gingipains), which constitute its main virulence factors. Two gingipains are specific

297 for Arg-Xaa peptide bonds (HRgpA and RgpB), whereas Kgp cleaves after a Lys residue

298 (Imamura, 2003). Interestingly, gingipains are involved in the disruption of host defense

299 inflammatory reactions and hinder *P. gingivalis* clearance by the immune system (Uehara et al.,

300 2008; Guo, Nguyen & Potempa, 2010). Human gingival fibroblasts play an important part in the

301 innate immune system by sensing microbial invasion and responding to it by producing and

302 secreting inflammatory mediators. HGFs recognize *P. gingivalis* during the early stages of

303 periodontitis and establish an inflammatory response in the periodontal tissue (Palm, Half &

304 Bengtsson, 2015). The secretion of TNF-α and IL-1β by HGFs favor the recruitment of

305 macrophages and neutrophils to the site of infection, as well as the expression of MMP-1, MMP-

306 13, MMP-8, and MMP-9, which contribute to the degradation of the extracellular matrix of the

307 periodontal tissue as well as the reabsorption of bone tissue (Ara et al., 2009; Song et al., 2021;

308 Cheng et al., 2020; Franco et al., 2017; Siu et al; 2020; Menaka et al., 2009).

309 Interleukin-1β (IL-1β), belongs to the IL-1 family and plays an important role against microbial

310 infections and participates regulating innate immune and inflammatory responses. The

311 upregulation of IL-1β during P. *gingivalis* infection suggests that IL-1β is a critical cytokine in

312   the host's defense against *P. gingivalis* infection during the initial phases of inflammation

313   (DInarello, 2009). In the early stages of *P. gingivalis* infection, IL-1β plays an important role in

314   combating the invading pathogen as part of the innate immune response and participates in

315   almost all events involved in the activation and regulation of inflammation (Menu & Vince,

316   2011). This kind of inflammasome-independent IL-1β activation can substantially contribute to

317   tissue inflammation (Latz & Xiao & Stutz, 2013).

318   We now demonstrate that cystatin C down-regulates the production of IL-1β and TNF-α in HFGs

319   co-incubated with *P. gingivalis*. Our finding is in accordance with the literature, where cystatin C

320   has been shown to down-regulate the production of IL-1β and TNF-α in monocytes stimulated

321   with bacterial LPS (Gren et al., 2016). In addition to cystatin C, other salivary antimicrobial

322   peptides, such as histatin 5 and histatin 1, also down-regulate inflammatory cytokines like IL-6,

323   IL-8, IL-1β, and TNF-α in fibroblasts and macrophages (Imatani et al., 2000; Lee et al., 2021).

324   Our data also show that cystatin C enhances IL-10 production by HFGs incubated with *P.*

325   *gingivalis*, which could represent an important mechanism to inhibit an excessive inflammatory

326   response of HGFs to the *P. gingivalis* infections. The cytokine IL-10 can inhibit pro-

327   inflammatory responses, due to its ability to reduce the production of TNF-α, IL-6, and IL-1

328   cytokines (Sun et al., 2020). Our results suggest that cystatin C could be an important

329   multifunctional modulator of the innate immune responses in HGFs.

330   In addition to cytokine production, HGFs also produce microbicidal mediators such as ROS and

331   NO, when they are infected with *P. gingivalis*. High doses of these molecules have been shown

332   to be cytotoxic to periodontal tissue (Nogueira et al., 2016), since their excessive production may

333   lead to tissue breakdown, including inhibition of energy-generating enzymes, triggering DNA

334   injury, oxidation and nitration reactions, (Wang, Huang & He, 2019; Bodis &

335   Haregewoin,1993). ROS causes oxidative damage to proteins and DNA, it interferes with cell

336   growth, and induces apoptosis in gingival fibroblasts, causing periodontitis (Kanzaki et al., 2017;

337   Cheng et al., 2015; Tomofuji et al., 2006; Marayuma et al., 2011). In addition to the damage

338   caused by ROS, an increase of iNOS expression and NO concentration also leads to severe

339   damage related to bone resorption, as shown in an experimental rat model of periodontitis

340   (Wang, Huang & He 2019). Thus, many inflammatory mediators are crucial for the development

341   of early periodontal disease, where NO is one of the main inflammatory factors (Pacher

342   Beckman, & Liaudet, 2007

343   Our data now demonstrated that *P. gingivalis* stimulates NO release by HGFs and that the co-

344   incubation of the bacterium with cystatin C significantly down-regulates both ROS and NO

345   productions. These findings are in accordance with the literature, showing that other peptides,

346   such as hBD3 and sublancin, also reduce the production of ROS in endothelial cells and NO in

347   peritoneal macrophages, respectively (Wang, Huang & He, 2019; Bian et al., 2017). The results

348   of our study suggest that NO expression could lead to the gradual progression of periodontitis

349   after proinflammatory cytokine production by HGFs infected by *P. gingivalis* and that cystatin C

350   protects from tissue damage through the reduction of these free radicals. The importance of ROS

351   in periodontal diseases was previously demonstrated by Cheng et al, who showed that LPS from

352   *P. gingivalis* up-regulated ROS in periodontal ligament fibroblasts (Cheng et al., 2015; Goltz et

353   al., 2014). The release of inflammatory mediators including interleukins, chemokines, adhesion

354   molecules, and ROS could be.could be triggered by bacteria LPS (Goraca et al., 2013; Melo et

355   al., 2010; Sanikidze et al., 2006; Bykov et al2003)

356   Antimicrobial peptides are included in the immune innate defense system in the oral cavity

357   (Greer, Zenobia & Darveau 2013). The antimicrobial peptide cystatin C belongs to the type 2

358   family of the cystatin superfamily, it is ubiquitously distributed in plants, animals, and

359   microorganisms (Shamsi & Bano, 2017). Saliva from the parotid gland of humans contains 0.9

360   μg/mL of Cystatin C (Gorr S, 2009). The main function of cystatin C is the inhibition of cysteine

361   proteases, by binding to their active sites, evading the cleavage of peptide bonds (van Wyk, et

362   al., 2016). The mechanisms leading to the reduction of the inflammatory mediators by cystatin C

363   are possibly explained by observations made with a homologous molecule, DsCistatin, isolated

364   from the tick *Dermacentor silvarum*. This peptide was shown to be internalized by endocytosis

365   in mouse macrophages stimulated with LPS from *Borrelia burgdorferi*. It reduced the

366   inflammatory cytokines IL-1β, IFN-γ, TNF-α, and IL-6 by the degradation of the TRAF6

367   protein, thereby preventing the phosphorylation of IκBα and the subsequent nuclear transport of

368   NF-κB, leading to the decrease of inflammatory cytokines (Sun et al., 2018). We speculate that

369   cystatin C possibly follows this route to reduce inflammatory mediators in HGFs incubated with

370   *P. gingivalis.*

371   Our data now show that cystatin C possibly plays an important antimicrobial and anti-

372   inflammatory role that regulates the response of human gingival fibroblast towards *P. gingivalis,*

373   helping to avoid tissue damage and destruction.

374

Conclusions

376 Cystatin C exhibits a dual activity during *P. gingivalis* infection. Antimicrobial activity was

377 demonstrated without cytotoxic effects on HGFs. Furthermore, Cystatin C also exhibited

378 immunomodulatory functions, decreasing the inflammatory response of fibroblasts. Knowledge

379 on the immunomodulatory properties of cystatin C could aid in the design of new therapeutic

380 approaches to improve the treatment of periodontal diseases.

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Acknowledgements

385 We thank Drs Daniela Cortés Hernández and Dulce Verónica Rivero Gamallo for their assistance

386 in the culture of bacteria and human fibroblasts during the initial phase of the study and Rocely

387 Cervantes Sarabia for her assistance in cytotoxicity assays.

388

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767 ADDITIONAL INFORMATION AND DECLARATIONS
768
769 Funding Ana María Fernández Presas
770 This work was supported by grant # IN218419, from PAPITT, DGAPA, UNAM, Mexico City,
771 and partially by Universidad Anahuac México Campus Norte . Blanca Esther Blancas-Luciano is

772   supported by CONACYT grant # 424031 for her doctoral studies. The funders had no role in study
773   design, data collection and analysis, decision to publish, or preparation of the manuscript.
774
775   Grant Disclosures The following grant information was disclosed by the authors:
776   PAPITT, DGAPA, UNAM, Mexico City: #IN218419.
777   CONACYT: # 424031

778
779   Competing Interests The authors declare there are no competing interests.
780
781   Author Contributions
782   Blanca Blancas, Jaime Zamora, Adriana Remigio, and Delgado Dominguez participate in the
783   conceptualization, study design, conducted all the experiments, Data curation, and reviewed
784   drafts of the paper
785   r, Leyva-Huerta ER, Portilla-Robertson J, participate in the conceptualization, data curation and
786   reviewed drafts of the paper.
787   I. Becker was involved in the concept of the study, Supervision, Visualization, critically revised
788   the manuscript, and approved the final draft,
789   A.M Fernández participated in Conceptualization, study design, conducted experiments,
790   supervision, visualization interpreted the data, drafted the manuscript, approved the final draft.
791   and acquired the funding.
792
793   Data Availability
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795   The following information was supplied regarding data availability:
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Figure 1

Effect of cystatin C on *Porphyromonas gingivalis growth* and cell viability.

Effect of cystatin C on **(A)** *Porphyromonas gingivalis* growth and **(B)** Cell viability of HGFs.
The results are expressed as mean ± SD of the average of five independent assays.
Statistical differences are expressed as (*) $p < 0.05$ when compared to non-treated control *P. gingivalis* bacteria.
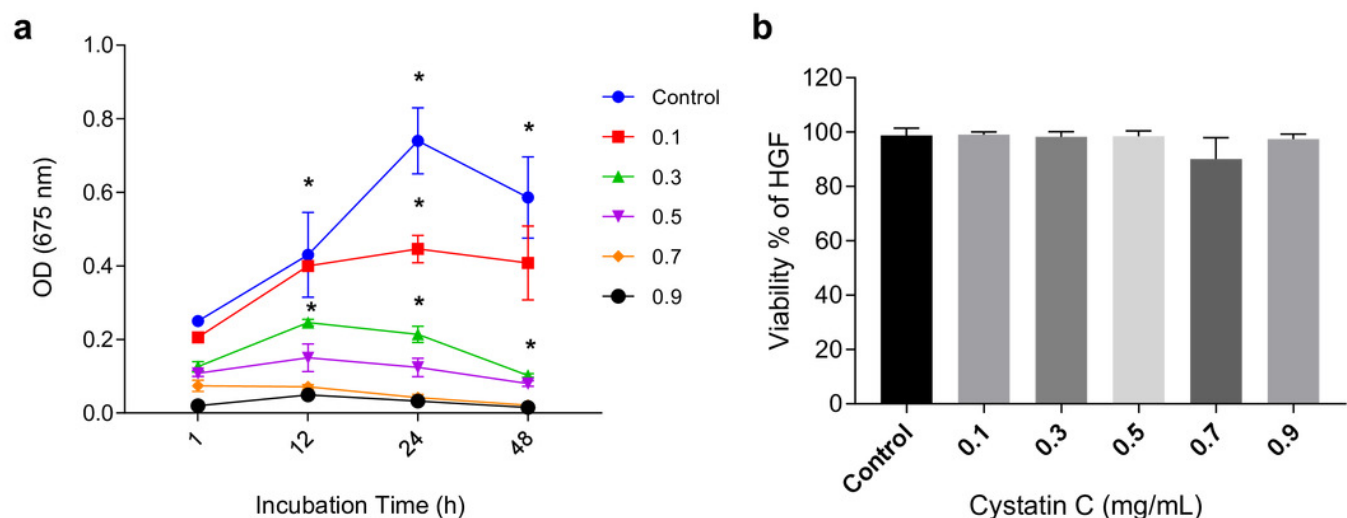
Figure 2

Cytokines assays in HGFs incubated with *P. gingivalis* and stimulated with cystatin C.

Cytokines assays in HGFs incubated with *P. gingivalis* and stimulated with cystatin C. **(A)** Expression of TNF-α, **(B)** IL-β, (**C**) IL-10. The results are expressed as mean ± SD of the average of five independent assays. Statistical differences are expressed as (*) $p < 0.05$, (**) $p < 0.001$, (***) $p < 0.0001$, compared to control groups.
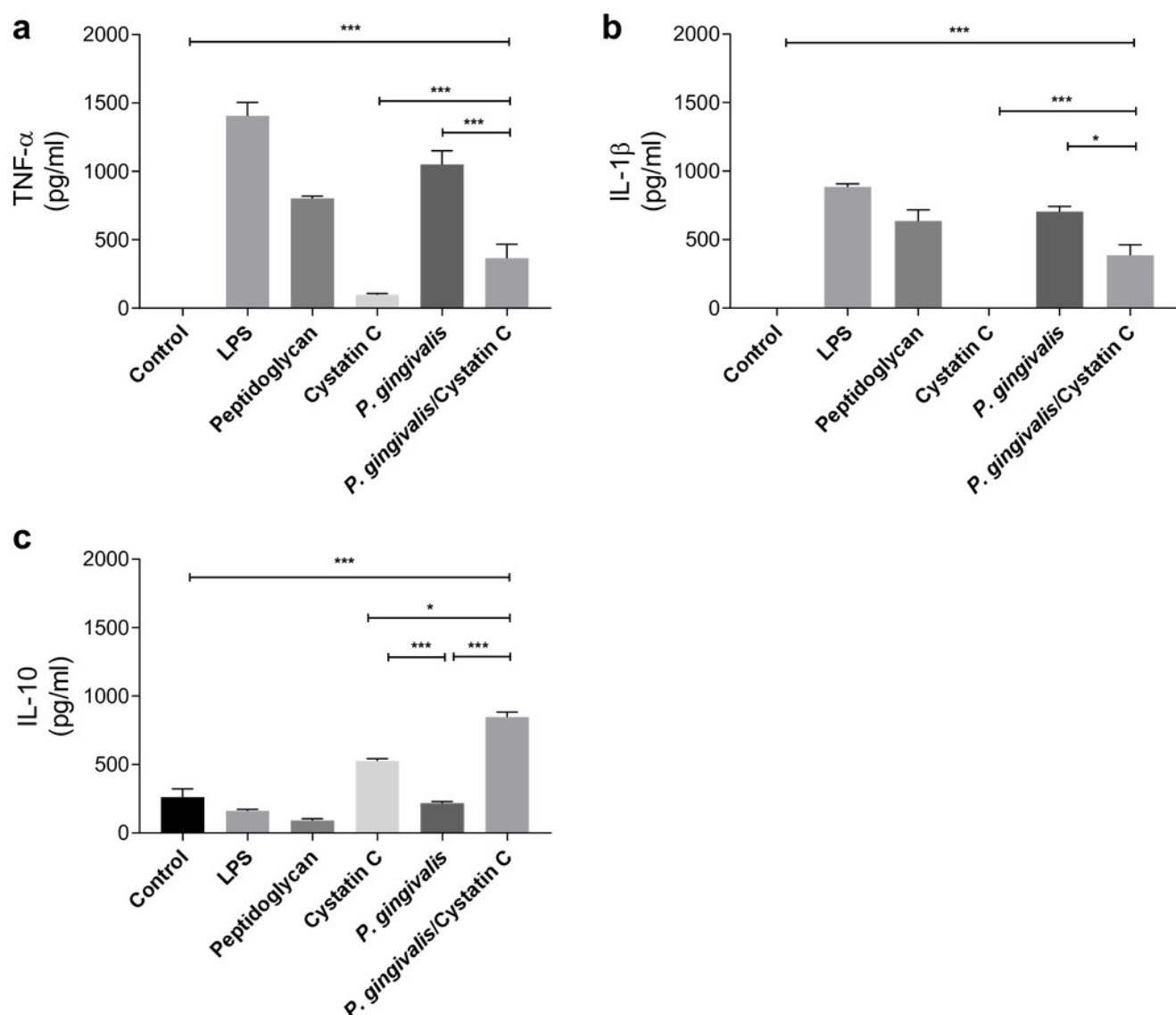
Figure 3

ROS and NO production *in Porphyromonas gingivalis* incubated with HGFs and cystatin C.

Porphyromonas gingivalis incubated with HGFs and cystatin C. **(A)** ROS production in HGFs infected with *P. gingivalis*. **(B)** Expression of nitric oxide in HGFs incubated with *P. gingivalis.* The results are expressed as mean ± SD of the average of five independent assays. Statistical differences are expressed as (*) p < 0.05, (**) p < 0.001, (***) p < 0.0001, compared to control groups.

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