Human dental pulp stem cells: a sanctuary for relapsing *Bartonella quintana*.

Hamadou Oumarou Hama [1,2*], Attoumani Hamada[1,2*], Gérard Aboudharam[1,2], Éric Ghigo[1,4] and Michel Drancourt[1]

[1] Institut Hospitalier Universitaire Méditerranée Infection, Marseille, France.

[2] Aix-Marseille-Université, IRD, MEPHI, IHU Méditerranée Infection, Marseille, France.

[3] UFR Odontologie, Aix-Marseille-Université, Marseille, France.

[4] Aix-Marseille Univ, IRD, AP-HM, SSA, VITROME, Marseille, France

These two authors equally contributed to this work.

Corresponding author :

Michel DRANCOURT

IHU Méditerranée Infection

19-21 Boulevard Jean Moulin

13385 Marseille, France

Phone: (+33) 4 13 73 20 51

Fax: (+33) 4 13 73 20 52

E-mail: michel.drancourt@univ-amu.fr

ABSTRACT

Bartonella quintana is a facultative intracellular bacterium responsible for relapsing fever, an example of non-sterilizing immunity. The cellular sanctuary of *B. quintana* in-between febrile relapses remains unknown but repeated detection of *B. quintana* in dental pulp specimens suggested long-term half-life dental pulp stem cells (DPSCs) as candidates. As the capacity of DPSCs to internalize microscopic particles was unknown, we confirmed that DPSCs internalized *B. quintana* bacteria: Gimenez staining and fluorescence microscopy localized *B. quintana* bacteria inside DPSCs and this internalization did not affect the cellular multiplication of DPSCs during a one-month follow-up despite the increase in the bacterial load. *B. quintana*-infected DPSCs did not produce Tumor Necrosis Factor-α whereas an important production of Monocytes Chemoattractant Protein-1 was observed. These unprecedented observations suggested the possibility that DPSCs were shelters for the long-term persistence of *B. quintana* in the host, warranting further experimental and clinical investigations.

Keywords: Human dental pulp stem cells, internalization, immunity, *Bartonella quintana*.

INTRODUCTION

Bartonella quintana is a facultative intracellular gram-negative bacterium described in 1915 as the agent of trench fever, emerging during World War I in soldiers presenting with fever, headache, sore muscles, bones and joints and skin lesions on the chest and back[1,2]. Trench fever is nowadays understood as one of the clinical forms of *B. quintana* bacteremia, also responsible for life-threatening endocarditis[3–6] and bacillary angiomatosis in immunocompromised patients[7]. Further, *B. quintana* is also responsible of lymphadenopathy in the lymphatic territory of its inoculation as *B. quintana* is an ectoparasite-borne pathogen transmitted from person to person by the body lice[1,8] and probably from cat to persons by cat fleas[9].

Trench fever is a relapsing fever and *B. quintana* has been consistently observed in circulating erythrocytes during febrile episodes[10] yet the site where *B. quintana* is residing in-between febrile episodes remains unknown even though the demonstration that bacillary angiomatosis results from the reactivation of quiescent *B. quintana* suggested such a role for endothelial cells as sanctuary cells[11,12]. However, neither erythrocytes nor endothelial cells have been demonstrated to host *B. quintana* for long, in agreement with the fact that both cell types have a limited life span time of 120 days for erythrocytes and 100 days for endothelial cells[13,14].

Interestingly, *B. quintana* has been consistently detected in the dental pulp, a highly vascularized organ with high erythrocyte trafficking[15]. As for an example, paleomicrobiology studies detected *B. quintana* with a prevalence of 2.5% to 21.4% in the dental pulp collected in buried populations[16,17]. In one particular burial site of Remiremont, the prevalence of *B. quintana* in 45 dental pulp specimens collected from these 5-10th populations, was as high as 53.3%[18]. Also, *B. quintana* has been

detected in the dental pulp collected from one patient who had been diagnosed with *B. quintana* bacteremia six months before tooth extraction and was free of bacteremia at the time of tooth extraction[19].

The dental pulp is composed of several cell types including dental pulp stem cells (DPSCs) which were investigated in the present study. DPSCs are mesenchymal stem cell isolated by Gronthos in 2000 and characterized by the expression of markers such as CD73, CD90 and CD105, whereas markers CD34 (hematopoietic progenitor cell antigen) and CD45 (leukocyte common antigen) are not expressed[20,21].

The DPSC stemness capacity correlates with a long lifespan[22,23] making them an attractive cell type to investigate hosting *B. quintana* for extended period compatible with clinical reports. In addition, DPSCs are located in the inner area of dental pulp chamber in close contacts with nerve ending and could be a sentinel cells for injury and blood-borne pathogen invasion. It has been found that DPSCs present an immuno-privileged against immune responses [24]. Indeed, DPSCs possess an immunomodulatory activity following LPS stimulation. They produce pro-inflammatory cytokines such as Interleukin (IL)-6, IL-8, Tumor Necrosis Factor (TNF)-α and Monocytes Chemoattractant Protein (MCP)-1 to recruit immune cell in the site of inflammation, and anti-inflammatory cytokines including IL-10 to reduce the inflammatory and maintain an homeostasis[25–27].

Based on this background, the aim of this present study was to investigate the role of DPSCs in host-pathogen interactions, using *B. quintana* as a paradigmal organism.

MATERIALS AND METHODS

Bacterial strain

B. quintana ATCC49793 was cultured on Columbia 5% sheep blood agar (COS) plates (bioMérieux, Craponne, France) at 37°C under a 5% CO_2 atmosphere. The identification of *B. quintana* was confirmed by matrix-associated laser desorption ionization/time of flight mass spectrometry (MALDI TOF MS) as previously described[28].

DPSCs culture.

After obtaining the patient's informed consent, a wisdom tooth was investigated in line with advice from the IHU Mediterranean Infection Ethics Committee (Advice, 05/29/2018). DPSCs obtained from this wisdom tooth were cultured in Dulbecco's Modified Eagle Medium F-12 (DMEM/F12, Invitrogen, Villebon-sur-Yvette, France) supplemented with 10% heat-inactivated foetal calf serum (FBS, qualified, EU-approved, South America origin, Gibco, Paisley, UK) at 37°C under a 5% CO_2 atmosphere. DPSCs viability was determined by using the Trypan blue exclusion assay. This assay is distinctively differentiating non-viable from viable cells based on the analysis of the integrity of the cell membrane[29]. Briefly, 50 µL of trypsinated DPSCs suspension were mixed with 50 µL of a 0.4% solution of Trypan blue dye (Eurobio, Les Ulis, France) for 1 min at room temperature. Cells were immediately counted using a Neubauer microchamber (Brand GmbH, Wertheim, Germany) with a light microscope using a 100 X magnification.

DPSCs infection.

B. quintana was collected in sterile tubes from two plates of COS and then washed twice in a row with sterile phosphate buffered saline (PBS). Infection of DPSCs (6 x 10^6) with *B. quintana* in cell culture medium was performed by centrifugation at 3,220 x g for 1 hour. The suspension was then distributed in flasks (SARSTEDT, Nümbrecht, Germany) (i.e. 2 mL per flask) and incubated at 37°C under 5% CO_2 atmosphere for a one month follow-up (12h, 24h, 48h, 72h, 1^{st}, 2^{th}, 3^{th} and 4^{th} week). DPSCs cultured alone and *B. quintana* were used as controls. After each incubation time, cells were washed thrice with sterile PBS and 200 µL of cell suspension were cytospined for 5 min (Shandon Cytospin 4, Thermo Scientific, Cheshire, UK). The identification of infected DPSCs was carried by Gimenez staining.

Fluorescent *in-situ* hybridization (FISH)

At the second week of infection a FISH was performed after cytospin and fixation of the slides with 4% paraformaldehyde for 20 min at room temperature. FISH was carried out as previously described with some modifications[30]. Briefly, probe 16S488-AATCTTTCTCCCAGAGGG labeled with Alexa-488 fluorochrome (Eurogentec, Angers, France) targeted *B. quintana* 16S rRNA gene. The cellular nucleus was stained in blue using 4',6-diamidino-2-phenylindole (DAPI, Fisher Scientific, Illkirch, France). Uninfected DPSCs were used as negative controls.

Cytokine quantification

The supernatants in the first week of the first passage of infected DPSCs and third, fourth weeks of infection were collected to evaluate the concentration of MCP-1 and TNF-α by using ELISA kits according to the manufacturer's protocols (R&D Systems,

139 Rennes, France), the mean minimum detectable dose of human MCP-1 was 1.7

140 pg/mL and 4.00 pg/mL for human TNF-α. The results were expressed in pg/mL.

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RESULTS

Capacity of DPSCs to internalize *B. quintana* bacteria

We first investigated whether DPSCs could internalize *B. quintana* bacteria. Gimenez staining allowing the staining of intracellular bacteria was our reference method. The validation of result was performed using a control of uninfected DPSCs and checking the form of *B. quintana* by Gimenez staining before infection **(Fig. 1)**. *B. quintana* effectively multiplies within DPSCs as indicated by the follow-up from 12 hours of incubation (**Fig. 2**). In addition, FISH was carried out in the second week of infection, confirming the presence of *B. quintana* within cells (in green) **(Fig. 3)**. The specificity of the probe was confirmed by a negative control (**Fig. 4**).

Immune response of DPSC to *B. quintana* infection.

We further investigated whether *B. quintana* internalization induced an immune pattern by DPSCs. *B. quintana* infection correlated with increased for MCP-1 with maximum of production in week four from 11175 to 19867.85 pg/mL (**Table 1)**. A production of MCP-1 was observed in supernatants of uninfected DPSCs but has a low concentration compared to the infected DPSCs and remains practically stable between the third and the four weeks of incubation (**Table 1)**. For TNF-α, we observed an absence of production suggesting an absence of pro-inflammatory responses TNF-α in infected DPSCs (Supplementary Table S1).

DISCUSSION

We observed that *B. quintana* was internalized by DPSCs. The infection with *B. quintana* did not affected cellular multiplication of DPSCs and despite the increase in these cells an increase in the numbers of bacteria was observed. Our observations

support the hypothesis that DPSCs could act as reservoir cells for *B. quintana*. This hypothesis is in accordance with a study reported that intracellular *B. quintana* bacteria could be internalized into a vacuolic compartment (*B. quintana*-containing vacuoles) and multiply[31–33]. These observations correlate with the persistence of *B. quintana* in human[34]. This is opening an exciting new role for DPSCs and further exploring their function could be done by additional observations including the co-localization of *B. quintana* and DPSCs in dental pulp specimens. If confirmed, the role of DPSCs as sanctuary cells for other pathogens will have to be investigated.

The absence TNF-α production suggests that *B. quintana* inhibits or alters the production TNF-α, most likely to favor its replication by avoiding the induction of the microbicidal activities of the DPSCs and the recruitment of pro inflammatory cells. Despite the absence of TNF-α production, production of MCP-1 was observed. This production of MCP-1 has already been described previously as being produced in large quantities by DPSCs[35] and did not induce DPSCs differentiation according to the literature[36,37]. In addition, it has been described that infection with the Chikungunya virus in human peripheral blood mononuclear cells induce a large production of MCP-1. However, suppression of MCP-1 does not affect replication of virus[38].

The role of MCP-1 remains unclear in *B. quintana* infection. Further investigations incorporating MCP-1 blocking antibodies may help defining the role of MCP-1 in the replication of *B. quintana* in DPSCs; but this experimental task was beyond the scope of the present study. Nevertheless, this study is opening a new venue for DPSCs as sanctuary cells for the long-term survival of relapsing pathogens.

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AUTHOR CONTRIBUTION STATEMENT

H.O.H. and A.H. performed the experiments, prepared figures. H.O.H., A.H., G.A., E.G. and M.D. designed the experiments, conceived the experiments, analysed the data, wrote the manuscript.

Conflict of interest

The authors have no conflicts of interest to declare. The funding sources had no role in the study design, data collection and analysis, decision to publish, or manuscript preparation.

REFERENCES

1. Maurin, M. & Raoult, D. *Bartonella (Rochalimaea) quintana* infections. *Clin Microbiol Rev* **9,** 273–292 (1996).
2. Byam, W. *et al.* Trench Fever. A Louse-Borne Disease. (Fowde, 1919).
3. Fournier, P. E. *et al.* Epidemiologic and clinical characteristics of *Bartonella quintana* and *Bartonella henselae* endocarditis: a study of 48 patients. *Medicine (Baltimore)* **80,** 245–251 (2001).
4. Raoult, D. *et al.* Diagnosis of 22 new cases of *Bartonella* endocarditis. *Ann. Intern. Med.* **125,** 646–652 (1996).
5. Drancourt, M. *et al. Bartonella (Rochalimaea) quintana* endocarditis in three homeless men. *N. Engl. J. Med.* **332,** 419–423 (1995).
6. Drancourt, M. *et al.* New serotype of *Bartonella henselae* in endocarditis and cat-scratch disease. *Lancet* **347,** 441–443 (1996).
7. Koehler, J. E. *et al.* Molecular epidemiology of bartonella infections in patients with bacillary angiomatosis-peliosis. *N. Engl. J. Med.* **337,** 1876–1883 (1997).
8. Badiaga, S. & Brouqui, P. Human louse-transmitted infectious diseases. *Clin. Microbiol. Infect.* **18,** 332–337 (2012).
9. Chomel, B. B. *et al.* Experimental transmission of *Bartonella henselae* by the cat flea. *J. Clin. Microbiol.* **34,** 1952–1956 (1996).
10. Rubin, L. G. 181 - Other Gram-negative coccobacilli. In *Principles and Practice of Pediatric Infectious Diseases (Fourth Edition)* (ed. Long, S. S.) 939-941.e1 (Content Repository Only!, 2012).
11. Greub, G. & Raoult, D. *Bartonella*: new explanations for old diseases. *J. Med. Microbiol.* **51,** 915–923 (2002).

12. Mosepele, M., Mazo, D. & Cohn, J. *Bartonella* Infection in immunocompromised Hosts: Immunology of vascular Infection and vasoproliferation. *Clinical and Developmental Immunology* **2012,** 1–5 (2012).
13. Castoldi, G. L. & Senno, L. del. Erythrocytes. In *Encyclopedia of Immunology (Second Edition)* (ed. Delves, P. J.) 833–841 (Elsevier, 1998).
14. Kliche, K., Jeggle, P., Pavenstädt, H. & Oberleithner, H. Role of cellular mechanics in the function and life span of vascular endothelium. *Pflügers Archiv - European Journal of Physiology* **462,** 209–217 (2011).
15. Maxim MA, Soritau O, Baciut M. et al. The role of dental stem cells in regeneration. *Clujul Med*. **88,** 479–482 (2015).
16. Tran, T.N., Forestier, C.L., Drancourt, M., Raoult, D., and Aboudharam, G. Brief communication: co-detection of *Bartonella quintana* and *Yersinia pestis* in an 11th–15th burial site in Bondy, France. *Am J Phys Anthropol*. **145**, 489–494 (2011).
17. Nguyen-Hieu, T. *et al.* Evidence of a louse-borne outbreak involving typhus in Douai, 1710-1712 during the war of Spanish succession. *PLoS ONE* **5,** e15405 (2010).
18. Drancourt, M., Tran-Hung, L., Courtin, J., Lumley, H. de & Raoult, D. *Bartonella quintana* in a 4000-year-old human tooth. *J. Infect. Dis.* **191,** 607–611 (2005).
19. Aboudharam, G. *et al.* Molecular detection of *Bartonella quintana* DNA in the dental pulp of a homeless patient. *Eur. J. Clin. Microbiol. Infect. Dis.* **23,** 920–922 (2004).
20. Dominici, M. *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8,** 315–317 (2006).

21. Gronthos, S., Mankani, M., Brahim, J., Robey, P. G. & Shi, S. Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proc Natl Acad Sci U S A* **97,** 13625–13630 (2000).

22. Allouba, M. H., ElGuindy, A. M., Krishnamoorthy, N., Yacoub, M. H. & Aguib, Y. E. NaNog: A pluripotency homeobox (master) molecule. *Glob Cardiol Sci Pract* **2015,** (2015).

23. Graziano, A., d'Aquino, R., Laino, G. & Papaccio, G. Dental pulp stem cells: a promising tool for bone regeneration. *Stem Cell Rev* **4,** 21–26 (2008).

24. Collart-Dutilleul, P.-Y., Chaubron, F., De Vos, J. & Cuisinier, F. J. Allogenic banking of dental pulp stem cells for innovative therapeutics. *World J Stem Cells* **7,** 1010–1021 (2015).

25. Zhao, Y., Wang, L., Jin, Y. & Shi, S. Fas Ligand Regulates the Immunomodulatory Properties of Dental Pulp Stem Cells. *J Dent Res* **91,** 948–954 (2012).

26. Attoumani, H., Drancourt, M. & Ghigo, E. Immune Properties of Human Dental Pulp Stem Cells and Interactions with the Immune System. **2,** 5 (2018).

27. Bindal, P., Ramasamy, T. S., Kasim, N. H. A., Gnanasegaran, N. & Lin, C. W. Immune responses of human dental pulp stem cells in lipopolysaccharide induced microenvironment. *Cell Biol. Int.* (2018). doi:10.1002/cbin.10938

28. Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM, Raoult D. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin Infect Dis*, 49:543-51 (2009). doi: 10.1086/600885

282 29. Samyuktha V, Ravikumar P, Nagesh B, Ranganathan K, Jayaprakash T, Sayesh
283 V. Cytotoxicity evaluation of root repair materials in human-cultured periodontal
284 ligament fibroblasts. *J Conserv Dent JCD.* 17:467–70 (2014).
285 30. Loukil A, Kirtania P, Bedotto M, Drancourt M. FISHing *Mycobacterium*
286 *tuberculosis* complex by use of a *rpoB* DNA probe bait. *J Clin Microbiol*. 2018; 56:
287 e00568-18, /jcm/56/10/e00568-18.atom.
288 31. Brouqui, P. & Raoult, D. *Bartonella quintana* invades and multiplies within
289 endothelial cells *in vitro* and *in vivo* and forms intracellular blebs. *Research in*
290 *Microbiology* **147,** 719–731 (1996).
291 32. Eicher, S. C. & Dehio, C. *Bartonella* entry mechanisms into mammalian host
292 cells. *Cellular Microbiology* **14,** 1166–1173
293 33. Zhang, P. *et al.* A family of variably expressed outer-membrane proteins (Vomp)
294 mediates adhesion and autoaggregation in *Bartonella quintana*. *Proc Natl Acad*
295 *Sci U S A* **101,** 13630–13635 (2004).
296 34. Foucault, C., Brouqui, P. & Raoult, D. *Bartonella quintana* characteristics and
297 clinical Management. *Emerg Infect Dis* **12,** 217–223 (2006).
298 35. Ahmed, N. E.-M. B., Murakami, M., Hirose, Y. & Nakashima, M. Therapeutic
299 Potential of Dental Pulp Stem Cell Secretome for Alzheimer's Disease Treatment:
300 An In Vitro Study. *Stem Cells International* vol. 2016 e8102478
301 https://www.hindawi.com/journals/sci/2016/8102478/ (2016).
302 36. Andrukhov, O. *et al.* Response of human periodontal ligament stem cells to IFN-γ
303 and TLR-agonists. *Sci Rep* **7**, (2017).
304 37. Srankova, J. *et al.* Pegfilgrastim and linagliptin potentiate chemoattraction of Ccr2
305 and Cd44 stem cells accompanied by alterations of cardiac Hgf, Igf-1 and Mcp-1 in
306 daunorubicin cardiomyopathy. *J Pharm Pharmacol* **71**, 1440–1450 (2019).

307 38. Ruiz Silva, M., van der Ende-Metselaar, H., Mulder, H. L., Smit, J. M. &
308 Rodenhuis-Zybert, I. A. Mechanism and role of MCP-1 upregulation upon
309 chikungunya virus infection in human peripheral blood mononuclear cells. *Sci Rep*
310 **6**, 32288 (2016).
311

FIGURE LEGENDS

Figure 1. Control of DPSCs and *B. quintana* by Gimenez staining before infection. (A) Form of *B. quintana* colored in red (B): uninfected DPSCs colored in green.

Figure 2. Monitoring DPSCs infection by *Bartonella quintana* (Gimenez staining).

(A): 12-hour inoculation (B): 24-hour inoculation (C): One-week inoculation (D): Four-week inoculation. *B. quintana* inside DPSCs.

Figure 3. Microscopic FISHing *B. quintana* into DPSCs. (A) DAPI filter (350nm) visualizes cells in blue via the detection of their nuclei (B) FITC filter (488nm) visualizes the 16S rRNA probe in green (C) merge of the two filters (DAPI and FITC).

Figure 4. Microscopic FISHing in negative control using the DAPI filter (350 nm) to visualize cells in blue via the detection of their nuclei (A) and FITC filter (488 nm) for the 16S rRNA probe (B).

TABLE LEGEND

Table 1. Quantitative determination of human MCP-1 concentrations (pg/mL in cell culture supernatants by ELISA.

Supplementary Table S1. Quantitative determination of human TNF-α concentrations (pg/mL) in cell culture supernatants by ELISA.

337 **Table 1.**

Supernatants		Concentration MCP-1 (pg/mL)
4th week	iDPSCs	19867.85
	DPSCs	5250
3th week	iDPSCs	12600
	DPSCs	5153,58
First passage to a week	iDPSCs	11175
	DPSCs	4800

338 iDPSC: *B. quintana*-infected DPSCs.

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341 Supplementary Table S1.

Standard curve		Samples		
(pg/mL)	O.D.	Supernatants		O.D.
1000	1.602	4th week	iDPSCs	0.103
500	0.877		DPSCs	0.101
250	0.538			
125	0.354	3th week	iDPSCs	0.104
62.2	0.22		DPSCs	0.099
31.3	0.159			
15.6	0.14	First passage to a week	iDPSCs	0.105
0	0.1		DPSCs	0.104

342 iDPSC : *B. quintana*-infected DPSCs. O.D. : Optical Density.

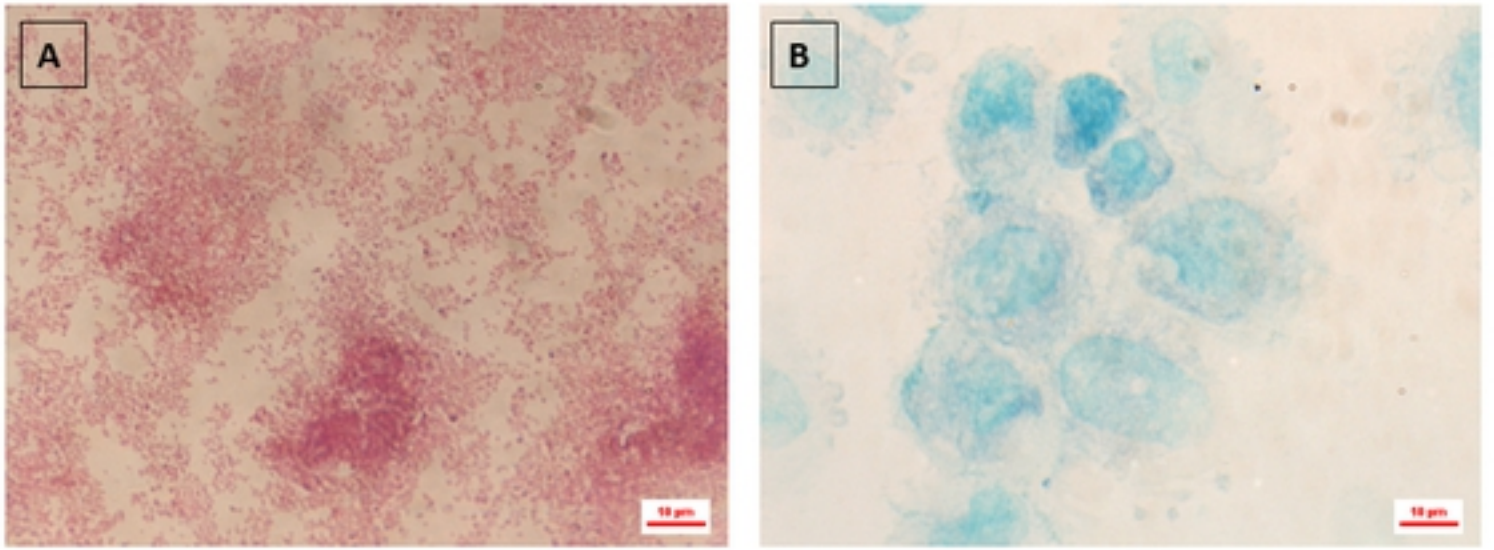

Figure 1

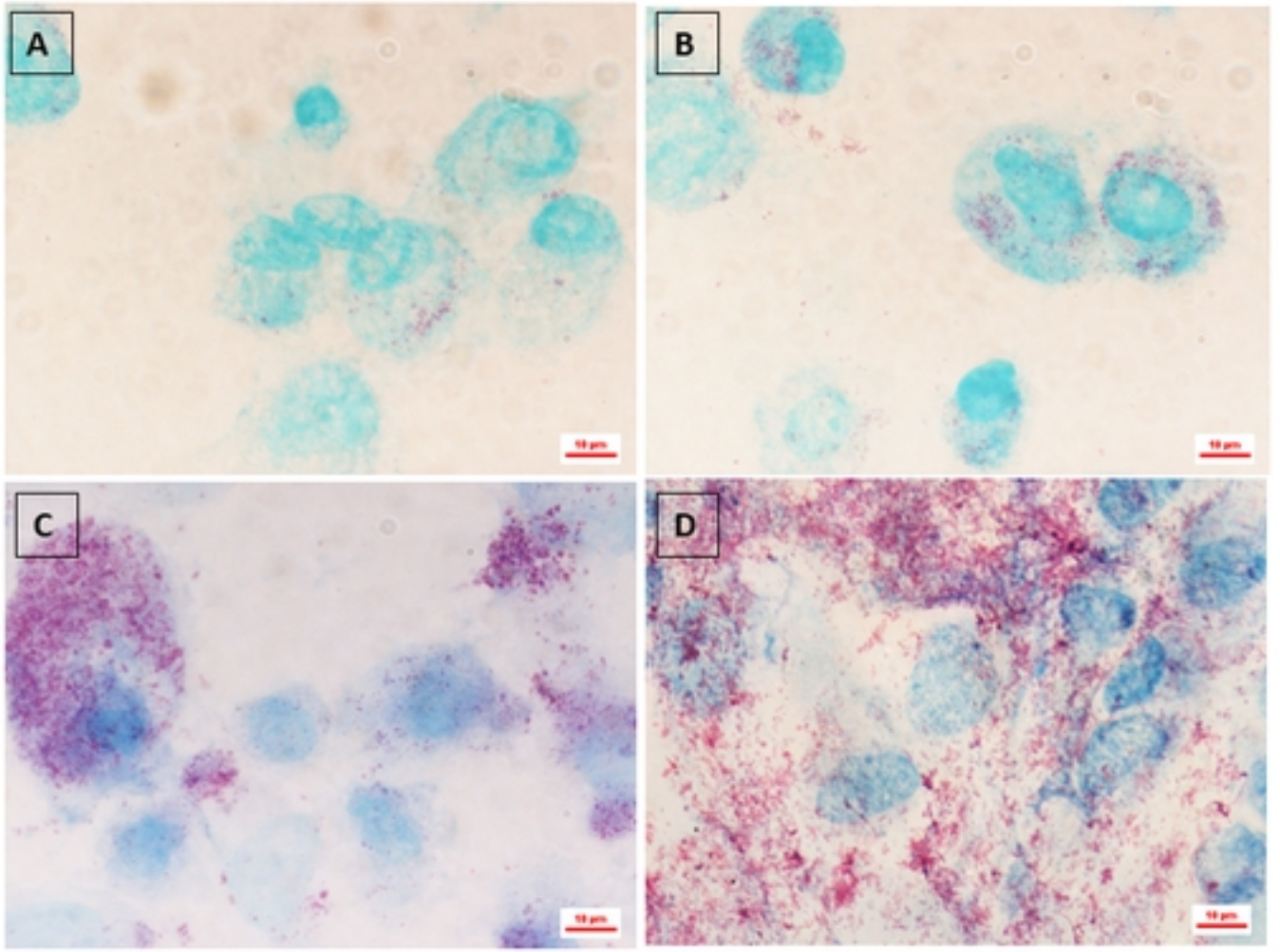

Figure 2

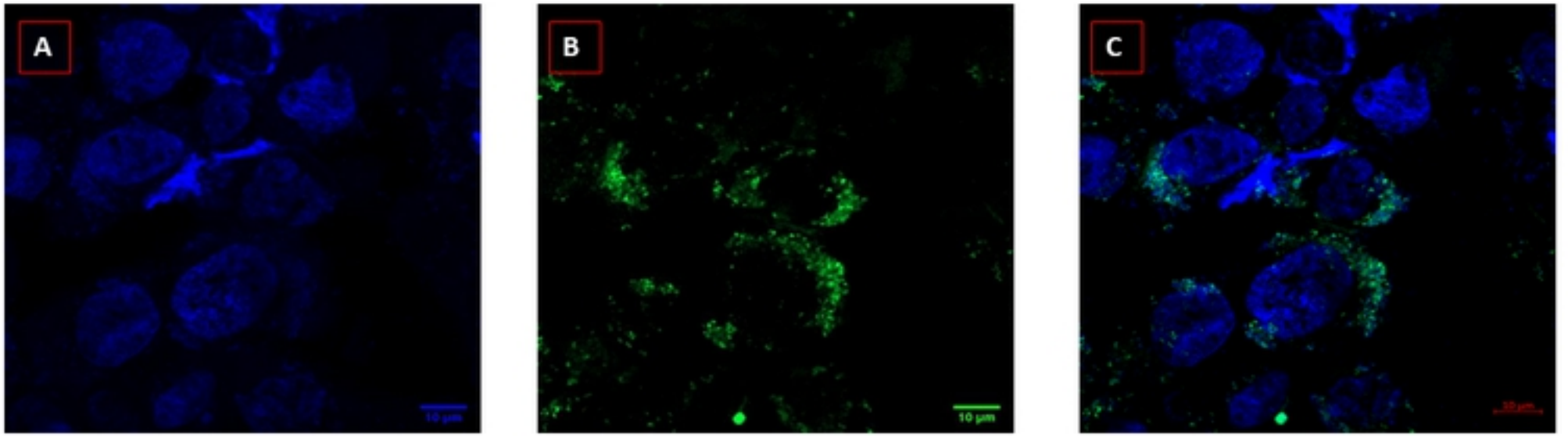

Figure 3

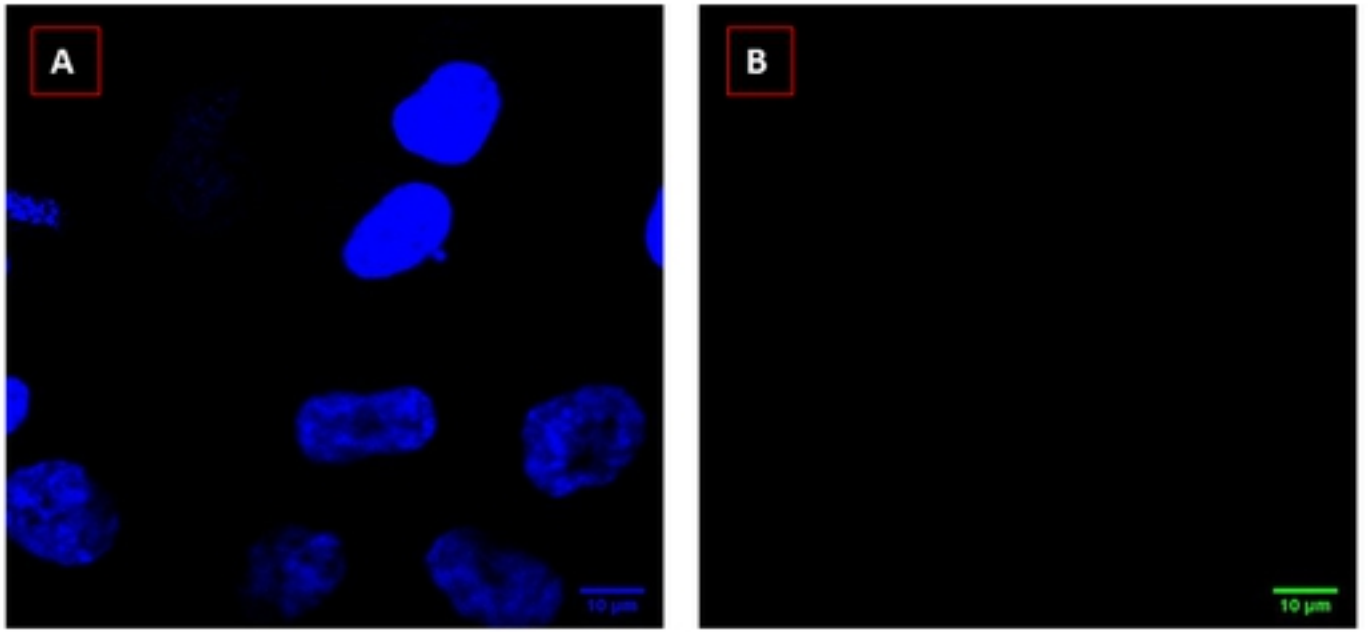

Figure 4