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Nupharidine Enhances *Aggregatibacter actinomycetemcomitans* Clearance by Priming Neutrophils and Augmenting Their Effector Functions

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Running title: Nupharidine primes neutrophils

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Abstract

Objectives: Nupharidine (6,6′-Dihydroxythiobinupharidine), purified from the aquatic plant *Nuphar lutea* leaves (Water lily) prompts antimicrobial activity of immune cells. The aim of the study was to test the effect of Nupharidine on neutrophil function against *Aggregatibacter actinomycetemcomitans*, JP2 clone (Aa-JP2).

Methods: Neutrophils derived from the human cell line HL60 and human peripheral blood derived from aggressive periodontitis and periodontally healthy subjects were incubated with Nupharidine or vehicle and inoculated with JP2. Bacterial survival was tested using viable counts on blood agar (CFU's). Neutrophils' necrosis/apoptosis, reactive oxygen species (ROS) production, phagocytosis and neutrophil extracellular traps (NET) production following infection were tested, as well as markers of neutrophil priming.

Results: Nupharidine had no direct bactericidal effect on JP2, but it enhanced Aa-JP2 clearance by neutrophils. Nupharidine enhanced neutrophil phagocytosis, ROS production and NET formation during JP2 infection. Furthermore, Nupharidine enhanced the expression of certain markers of neutrophils priming, specifically iCAM1, DECTIN-2 and intracellular IL-1β.

Conclusion: Nupharidine was shown to promote neutrophil effector bactericidal functions, boosting Aa-JP2 clearance. The results point to the potential of Nupharidine as an adjunctive agent in the treatment of Aa-JP2 periodontitis, but this should be tested initially using pre-clinical and clinical studies.

Clinical relevance

Scientific rational for the study: We have previously shown that *Nuphar lutea* semi-purified extracts have anti-inflammatory, anti-cancer and anti-leishmanial activity, as well as the ability to modulate NF-kB activation, cytokine and iNOS production. The current study aimed to investigate whether purified Nupharidine can promote neutrophil anti-microbial functions during inoculation with the aggressive periodontitis pathogen *Aggregatibacter actinomycetemcomitans*, clone JP2 *in-vitro*.

Principal findings: Nupharidine had no direct bactericidal effect on Aa-JP2 but significantly reduced counts of viable JP2 in the presence of neutrophils. All tested Neutrophilic antibacterial effector functions (ROS production, NET formation and Phagocytosis) were augmented by Nupharidine, as well as increased expression of markers of neutrophil priming.

Practical implications: Nupharidine has the potential to enhance bacterial clearance in Aa-JP2 periodontitis and, as such, may assist in the treatment of the disease.

Introduction

Neutrophils play a crucial role in the clearance of pathogens, by various effector functions such as phagocytosis, degranulation and formation of neutrophil extracellular traps (NETs) (Scott and Krauss, 2012). Periodontitis in adolescents and young adults (formerly Aggressive Periodontitis) is associated with specific infection by *Aggregatibacter actinomycetemcomitans*, JP2 clone (Aa-JP2) (Haubek et al., 2008) and also with abnormal neutrophil function (Scott and Krauss, 2012, Shapira et al., 1991, DeNardin et al., 1990, Sigusch et al., 2001, Suzuki et al., 1984). Classical studies have demonstrated that in this variant of periodontitis, neutrophils have impaired chemotaxis (DeNardin et al., 1990, Sigusch et al., 2001), defective phagocytosis (Suzuki et al., 1984) and increased superoxide production (Shapira et al., 1991). These impaired functions may explain why the host is unable to effectively resolve Aa-JP2 infection. Further, Aa-JP2 holds powerful virulence factors, such as the exotoxins - cytolethal distending toxin and leukotoxin A, of which the later causes neutrophils death via necrosis and evasion from phagocytosis (Claesson et al., 2002, Johansson et al., 2000).

Clinical treatment of Aa-JP2 periodontitis has historically proved to be challenging due to the fact that depletion of this keystone pathogen is poorly achieved by non-surgical treatments. Consequently, concomitant use of antibiotics or surgical debridement is included to improve treatment outcomes [3]. This clinical reality prompts the search of new strategies to treat the disease. Furthermore, a great concern over bacterial antibiotic resistance supports the development of alternative treatment modalities excluding antibiotic regimens.

Unlike standard treatment modalities, emerging data reveals the potential of unconventional medicine for managing infectious disease. Such modalities are based on various Plant extracts, phytochemical compounds and essential oils. In the periodontal field, the use of natural products has scarcely been

investigated. Such studies showed various biological effects mainly on bacterial physiology. *Vaccinium macrocarpon* (Ericaceae) fruit (cranberry) is one of the most studied anti-bacterial adhesion herbs. Flavonoids and proanthocyanidins isolated from fresh cranberry fruit were found to inhibit growth and biofilm formation of *P. gingivalis* (Feghali et al., 2012, Polak et al., 2013). *Camellia sinensis* (Theaceae) is another natural product originating in China. Green tea made from *C. sinensis*, and its polyphenols potently blocked in vitro the adhesion of *P. gingivalis* and have been shown to inhibit dental plaque accumulation (Sakanaka et al., 1996). *Kaempferia pandurata* Roxb. (Zingiberaceae), known as temu kunci in Indonesia, has been used traditionally for food and medicinal purposes, and its bioactive components - Panduratin A and isopanduratin A exhibit strong activities against cariogenic pathogens and periodontal pathogens, especially *P. intermedia* and *P. gingivalis* (Yanti et al., 2009). *Eugenia caryophyllata* L. (Myrtaceae) extract is another widely used natural product and is active against oral bacteria associated with periodontal disease (Cai and Wu, 1996) as well as a large number of other pathogenic bacteria (Chaieb et al., 2007). Other active constituents of clove (biflorin, kaempferol, rhamnocitrin, myricetin, gallic acid, ellagic acid and oleanoic acid) showed antibacterial activities against Gram-negative anaerobic periodontal oral pathogens, including *P. gingivalis* and *P. intermedia* (Cai and Wu, 1996). The *Eucalyptus* (Myrtaceae) tree extracts were shown to have specific activity against periodontal bacteria, such as *P. gingivalis*, *A. actinomycetemcomitans* and *Fusobacterium nucleatum* (Takarada et al., 2004).

Several plant organs of *Nuphar lutea* (Yellow Water Lilly) are used in traditional medicine for the treatment of arthritis, fever, aches, pains and inflammation (Ozer et al., 2009). We showed that semi-purified extracts of *Nuphar lutea* containing active thio-sesquiterpene molecules, Nupharidine (6,6'-Dihydroxythiobinupharidine), have anti-cancer and pro-apoptotic activity (Ozer et al., 2009, Ozer et al., 2017), as well as anti-inflammatory (Ozer et al., 2015) and modulatory effects on NF-κB (Ozer et al.,

2009). In addition, it has anti-leishmanial activity (El-On et al., 2009) and increased iNOS production by macrophages, against *Leishmania major* (Ozer et al., 2010). Nupharidine was also shown to have anti-microbial properties against multidrug resistant bacteria (Okamura et al., 2015).

The current study investigated the *in vitro* anti-microbial capability of Nupharidine against *A. actinomycetemcomitans* JP2 and its effect on neutrophils. The effect of Nupharidine was determined on HL-60 derived neutrophils, and human peripheral blood neutrophils derived from young patients with periodontitis and periodontally healthy volunteers.

Materials and Methods

Neutrophil-like cells

Neutrophil-like cells were differentiated from the HL-60 cell line (ATCC, Manassas, USA), with 1.3% dimethyl sulfoxide (DMSO) for 4 days as detailed by Collins et al (Collins et al., 1978). Cells were incubated at 37^{o}C, 5% CO_2 in 1640 RPMI medium with L-glutamine (20nM), and 10% heat inactivated fetal calf serum (FCS), Penicillin (100U/ml) and Streptomycin (100 µg/ml) (Biological Industries, Beit Haemek, Israel).

Primary neutrophils were also obtained at the morning of the experiment from venous blood samples taken from patients according to their periodontal diagnosis (n=3 for each group): periodontal healthy volunteers or young patients with classical symptoms of previously diagnosed and treated Localized Aggressive Periodontitis (LAP, molar-incisor type) according to the 1999 AAP criteria (1999)). Blood was collected in standard heparin tubes, and neutrophils were isolated using Polymorphprep (Axis-shield, Oslo, Norway) according to manufacturer instructions.

The study was approved by the institutional Helsinki review board (Hadassah Medical Center approval Number HMO-0514-13.

Bacteria

A. actinomycetemcomitans JP2 clone (clinical isolate, kindly provided by Prof. Gilad Bachrach, Hebrew University) was grown in a medium containing 0.5g Yeast extract, 1.5g Tryptone, 0.74g D-glucose, 0.25g NaCl, 0.075g L-cysteine, 0.05g Sodium thioglycolate and 4% $NaHCO_3$ (all purchased from Sigma-Aldrich, Rehovot, Israel) in double distilled water. JP2 purity was validated using TVSB selective culture (Slots, 1982) and PCR of bacterium` leukotoxin promoter region (Hoglund Aberg et al., 2014). Bacteria were grown for 24 hrs at 37°C in a 5% CO_2 chamber. Bacterial concentration was standardized by optical density according to Mattiello et al. (Mattiello et al., 2011). All experiments were done at multiplicity of infection 10.

Nupharidine

Nupharidine (6,6'-Dihydroxythiobinupharidine) was purified from *Nuphar lutea* leaves and was purchased from Sigma-Aldrich/Merck (Cat. SMB00609). A stock solution in DMSO was made, and working dilutions were made in phosphate buffered saline (PBS). Solutions were kept at -20°C.

Bacterial survival

Aa-JP2 were incubated for 1 hour with Nupharidine or vehicle control in the presence or absence of neutrophils ($5X10^5$ cells/well). After incubation supernatants were seeded on blood agar in 10-fold dilutions and incubated for 7 days at 37°C in a 5% CO_2 chamber, after which Colony Forming Units (CFU) were calculated.

Neutrophils cytotoxicity

Neutrophils were seeded in 12 well plates ($5X10^5$ cells/well) with Nupharidine (or vehicle) in different concentrations in a neutrophils antibiotic free medium for 1hr. The cells were then stained with Annexin V/Propidium iodide (Biolegend, San Diego, CA, USA) according to manufacturer instructions to determine levels of apoptosis and necrosis. Cell analysis was done by Flow cytometry (BD Accuri™ C6 Cytometer, San Jose, CA, USA).

Phagocytosis

Neutrophils ($5X10^5$ cells/well) where incubated with Nupharidine or vehicle and fluorescein isothiocyanate (FITC)-stained JP2 (Polak et al., 2017) for 1hr.

For microscopic images, after incubation cells underwent fixation and permeabilization using the Cytofix/Cytoperm™ kit (BD, San Jose, CA, USA) for 30 minutes, internal staining with PE conjugated LAMP1 antibody (Abcam, Cambridge, UK) and nuclear (DAPI) staining. Microscopic images were taken using a florescent microscope (Olympus BX43, Tokyo, Japan).

Quantitative analysis was performed on cells which underwent quenching using 0.2% trypan blue and analysis by flow cytometry (BD Accuri™ C6 Cytometer, San Jose, CA, USA). The percentage of cells positive for FITC (Neutrophils with FITC labeled bacteria within cell walls) was calculated.

Reactive oxygen species (ROS)

Neutrophils ($5X10^4$ cells/well) were incubated with Nupharidine or vehicle in PBS medium supplemented with calcium and magnesium in black 96-well plates (PerkinElmer, Waltham, MA). 0.5µg in 50µl PBS of 7'-dichlorofluorescein diacetate (Sigma-Aldrich, Israel) was added to each well together with JP2 bacteria. The plate was incubated in a florescence plate reader (infinite plate reader, Tecan, Männedorf, Switzerland) at 37^{o}C with readings at 490nm excitation and 535nm emission, every 5min for a total of 90min. Following the readings, the cells were washed in PBS and transferred into 0.5% bovine serum albumin (Sigma, Rehovot, Israel) in PBS and analyzed by flow cytometry for intracellular ROS.

Neutrophil extracellular trap (NET) formation.

Neutrophils ($5X10^4$cells/well) where incubated with Nupharidine or vehicle and JP2 for 3 hours in a 96-well plate. After incubation, cells were washed from unbound DNA and bacteria. Membrane bound extracellular DNA was detached using micrococcal nuclease (Worthington, Lakewood, NJ) and transferred to a black 96 well plate (PerkinElmer, Waltham, MA). The detached DNA was then stained with Sytox green (Invitrogen, Carlsbad, CA) and quantified with a florescent plate reader (infinite plate

reader, Tecan, Männedorf, Switzerland) (Hirschfeld et al., 2016).

Staining of neutrophil markers

Surface antigen staining

Treated neutrophils (HL60) were incubated with either CD54 (iCAM1) Rat monoclonal antibody conjugated to Phycoerythrin (Abcam, Cambridge, UK) or with Dectin-2 mouse monoclonal antibody (Abcam, Cambridge, UK) and secondary goat polyclonal anti-Mouse IgG1-heavy chain antibody conjugated with Allophycocyanin (Abcam, Cambridge, UK). Cell analysis was done with a flow cytometer (C6 BD Accuri™ C6 Cytometer, San Jose, CA, USA).

IL-1β, P50 and P65 intracellular staining

Treated neutrophils (HL60) were permeabilized in 100µl Cytofix/Cytoperm™ kit (BD, San Jose, CA, USA) for 30 minutes followed by incubation with either rabbit polyclonal anti-IL-1β (Abcam, Cambridge, UK). The cells were then washed with PBS and incubated with a secondary goat anti-rabbit or mouse IgG conjugated to FITC (Abcam, Cambridge, UK) for 30 minutes in ice and washed with PBS. Analysis was done using a flow cytometer (C6 BD Accuri™ C6 Cytometer, San Jose, CA, USA).

Data analysis

All experiments were performed in triplicates and were repeated at least three times. The data was analyzed using a statistical software package (SigmaStat, Jandel Scientific, San Rafael, CA, USA). One-way repeated measure analysis of variance (RM ANOVA) was applied to test the significance of the differences between the treated groups. If the results were significant, inter-group differences were tested for significance using Student's t-test and the Bonferroni correction for multiple testing.

Results

Nupharidine`s effect on Aa-JP2 survival following incubation with HL-60 derived Neutrophils

Based on previous cytotoxic data on cell lines (Ozer et al., 2009, Ozer et al., 2017, El-On et al., 2009) and validation on neutrophils (Figure 1A), two non-toxic concentrations of Nupharidine (12.5 and 25 µg/ml), were tested on neutrophils. Viability analysis demonstrated approximately 6% non-vital cells (negative for both apoptosis and necrosis markers) in the control, and 6.41% and 8.39% non-vital cells in the 12.5µg and 25µg Nupharidine treated cells respectively. When neutrophils were infected with Aa-JP2 (Fig 1B) Nupharidine led to approximately 80% decrease in Aa-JP2 survival compared to controls (vehicle only), without a difference between the 2 tested concentrations (Figure 1B). Neutrophils viability following infection with Aa-JP2 did not change in the presence of Nupharidine compared with infected neutrophils without Nupharidine (supplementary figure 1).

Further experiments were undertaken using the lower Nupharidine concentration that demonstrated effectiveness (12.5µg/ml).

Direct antibacterial effect of Nupharidine on JP2 viability

We examined the antibacterial effect of Nupharidine by incubating Aa-JP2 with Nupharidine (12.5µg/ml) or vehicle. Live bacteria were determined by CFU analysis. The results showed that treatment with Nupharidine had no direct bactericidal activity on Aa-JP2 under the conditions tested (data not shown).

Nupharidine`s effects on HL-60 derived neutrophils' killing mechanisms

Since Nupharidine did not show direct bactericidal properties against Aa-JP2, the most obvious supposition is that the enhanced bacterial killing in the presence of neutrophils is mediated by boosting

neutrophil functions. Therefore, subsequent experiments focused on neutrophil effector functions - phagocytosis, reactive oxygen production (ROS) and generation of extracellular traps.

Phagocytosis – Neutrophils engulf Aa-JP2 in their phagosome following Nupharidine treatment, portraying amplification of Aa-JP2 phagocytosis in the presence of Nupharidine compared with vehicle control (Fig 2A). Quantification of phagocytic cells revealed a significant increase after incubation with Nupharidine, and 71.2% of cells engulfed Aa-JP2 after Nupharidine treatment in comparison to only 19.8% of controls (p=0.002, Fig 2B). However, the amount of engulfed bacteria per cell (measured as geometric mean florescence) was not significantly different between groups (Fig 2C).

Reactive oxygen production (ROS) - Total ROS (intracellular and extracellular) was found to be mildly elevated in the Nupharidine exposed neutrophils with Aa-JP2 ($P<0.001$, Figure 3A). Interestingly, analysis of intra-cellular ROS indicated that Nupharidine significantly augments internal ROS compared with controls ($P=0.038$, Figure 3B).

Neutrophil extracellular traps - NET production was also significantly higher in the Nupharidine group vs. control ($P<0.001$, Figure 4).

Nupharidine effect on primary neutrophils from diseased and healthy subjects

In order to confirm the results with HL-60 derived neutrophils, we also performed experiments on primary human neutrophils obtained from peripheral blood of LAP—patients and periodontally healthy subjects, as described in the methods section. We exposed the cells to Aa-JP2 with or without the presence of Nupharidine

Decreased bacterial survival and increased ROS production was observed in both primary cell sources after incubation with Nupharidine (Figure 5) compared to neutrophils without Nupharidine. The cells

from diseased patients were more responsive to Nupharidine treatment than those derived from healthy volunteers (Figure 5A). These results were similar to those obtained with HL-60 derived cells.

Enhancement of priming markers expression in HL-60 derived neutrophils by Nupharidine

The above results indicate that Nupharidine enhanced neutrophil effector cell functions (phagocytosis, ROS and NETosis), suggesting priming-like agent properties of Nupharidine. Therefore, expression of specific markers of neutrophil priming: iCAM and Dectin-2, as well as intracellular expression of IL-1β were tested (Yao et al., 2015).

Surface antigens – Treatment with Nupharidine significantly augmented the expression of outer membrane iCAM and Dectin-2 compared with vehicle control samples (Figure 6). Quantitative analysis of iCAM and Dectin-2 expression was significantly greater in the Nupharidine group compared with controls (Figure 6).

Cytokine production – Neutrophils treated with Nupharidine showed increased expression of IL-1β (Figure 6). Control neutrophils (vehicle) also expressed IL-1β, but to lesser extent than their level in the test group (Figure 6). Quantitative analysis of IL-1β revealed a statistically significant difference between Nupharidine and control (Figure 6).

Discussion

Neutrophils are one of the first immune cells to react to a periodontal infection. An aggressive from of periodontitis in young adults is characterized by changes in neutrophil functions and persistent infection with the JP2 clone of *A. actinomycetemcomitans* [1-5]. The current study tested the effect *Nuphar Lutea*

extract, an anti-microbial and anti-inflammatory agent (El-On et al., 2009, Ozer et al., 2015, Turker H., 2009), on the interaction between neutrophils and Aa-JP2.

The purified active ingredient of *N. Lutea* extract, 6,6'-Dihydroxythiobinupharidine ('Nupharidine'), exhibited enhanced clearance of Aa-JP2 by neutrophils with little change in the viability of the neutrophils. On the other hand, a previous study showed that *Nuphar Lutea* leaf extract inhibits NF-κB pathways and can induce apoptosis in Hodgkin's lymphoma and breast carcinoma cell lines (Golan-Goldhirsh, 2008). The reason for this discrepancy likely stems from the fact that neutrophils are fully differentiated cells and do not resemble the behavior of cancerous cells.

In the current study we found that Nupharidine had no direct anti-bacterial effect on *Aa*-JP2. *Nuphar Lutea* extracts were found to have a direct leishmanicidal effect on *Leishmania major* (El-On et al., 2009) and an indirect effect through the activation of macrophages (Ozer et al., 2010). In another study, Turker et al. (2009) found an antibacterial effect of *N. Lutea* extracts on several bacterial strains isolated from fish (Turker H., 2009) and Okamura et al. (2015) reported similar results against multidrug resistant bacteria (Okamura et al., 2015). These results are in line with the fact that Nupharidine has a selective anti-microbial effect on certain micro-organisms while other microbes may be resistant to its effect (Turker H., 2009, Okamura et al., 2015). In light of the absence of a direct effect of Nupharidine on JP2 viability, the bacterial clearance observed with Nupharidine and neutrophils infected with JP2, stems from an augmented ability of neutrophils to kill this pathogen. Still, one cannot exclude the possibility that Nupharidine may impact Aa-JP2 leukotoxicity, which in turn affects the interaction between the pathogen and neutrophils. Indeed, Nupharidine may affect Aa-JP2 toxicity by neutralization of JP2 leukotoxicity, similar to serum neutralization (Tsai et al., 1981).

Subsequent data revealed a significant increase in all tested neutrophil effector mechanisms: (a) Nupharidine led to a mean increase of 55.1% in cells containing engulfed JP2; (b) Total ROS production was increased by Nupharidine by a mean of 26.5% in the presence of JP2; (c) Nupharidine led to a 51%

increase in neutrophils displaying increased intracellular ROS production; (d) Nupharidine treatment increased NET production against JP2 by 106%.

Taken together, the results regarding ROS (with significant elevation in intra-cellular ROS) and phagocytosis, leads to the conclusion that Nupharidine enhances the clearance of Aa-JP2 by phagocytosis and its killing via intracellular ROS. These results were confirmed using primary neutrophils derived from healthy volunteers and periodontitis patients. ROS excess, which causes oxidative stress, can result in cell damage and pathologies such as atherosclerosis, diabetes, cancer, neurodegeneration and ageing (Ray et al., 2012). Furthermore, it has a clearly defined and substantial role in periodontal tissue destruction and periodontitis (Chapple, 1997) as ROS causes de-polymerization of hyaluronic acid and to a lesser extent proteoglycan, which are both constituents of extracellular matrix in the periodontium (Bartold et al., 1984). In the current research, we show that total ROS levels were slightly elevated but a more substantial difference was detected regarding internal ROS production. We conclude that the secretion of ROS extracellularly was only slightly elevated after Nupharidine treatment, suggesting an antioxidative effect of Nupharidine with minimal likely danger to surrounding tissues following its use *in vivo*.

Neutrophil extracellular traps play a role in the development of several autoimmune diseases: components of NETs were found to cause an autoimmune response in small vessel Vasculitis; In Systemic Lupus Erythematosus (SLE), NETs are considered a possible source of autoantigens due to its co-occurence with impaired degradation by DNase 1 (Yang et al., 2016); NETs provide a scaffold and stimulus for platelet and red blood cell adhesion and aggregation to blood vessel walls and therefore promote thrombosis (Fuchs et al., 2010); A key factor in Rheumatoid Arthritis (RA) is an autoimmune response against citrullinated proteins. Citrullination of proteins is a part of NET formation and therefore link NETosis to RA (Spengler et al., 2015); NETs are also currently studied in correlation to tumor formation, sepsis and their amplification in diabetic patients (Yang et al., 2016). On the other

hand, it may increase neutrophil anti-bacterial activity, via DNA-bound antimicrobial proteins and peptides (Brinkmann et al., 2004). The induced NETosis during Aa-JP2 infection found in the current study is in accordance with Hirschfeld et al. who showed that Aa-HK 1651 (Member of the clone JP2) or its leukotoxin induce neutrophils` NETosis (Hirschfeld et al., 2016). Still, whether the increase neutrophils NETosis and ROS production by Nupharidine have detrimental or protective effect on the periodontal tissues requires further *in vivo* research (White et al., 2016).

The response of diseased patients` primary neutrophils versus periodontally healthy neutrophils without Nupharidine reflects the well-established neutrophils hypo-function associated with aggressive periodontitis (Scott and Krauss, 2012, Shapira et al., 1991, DeNardin et al., 1990, Sigusch et al., 2001, Suzuki et al., 1984). The beneficial effect of Nupharidine on the diseased patients' primary neutrophils may suggest that the Nupharidine priming of diseased patients` neutrophils recover neutrophils function to the functional levels of healthy neutrophils infected with Aa-JP2.

In accordance with the findings of Yao et al. (Yao et al., 2015) we found that neutrophils exhibit enhanced production of intracellular IL-1β immediately after being treated with Nupharidine, and presented surface markers CD54/iCAM and Dectin-2 after treatment with Nupharidine. These results indicate that Nupharidine enhances Neutrophils' anti-microbial activity by priming neutrophils. This occurs naturally in sites of inflammation. In fact, the migration of neutrophils into tissues is enhanced by priming (Yao et al., 2015). Therefore, priming is likely to occur in an *in vivo* situation.

In conclusion, Nupharidine *in vitro* was found to have no direct bactericidal effect on *Aggregatibacter actinomycetemcomitans* JP2 clone, but it enhanced JP2 clearance by neutrophils. The enhanced clearance was associated with upregulation of neutrophil killing mechanisms, and priming of the neutrophils. Still, *in vivo* animal models as well as clinical trials are required to validate the results.

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Figure legends

Fig 1 –cytotoxic effect of Nupharidine on JP2 and neutrophils

A - Nupharidine (12.5μg/ml or 25 μg/ml) cytotoxicity against HL-60 derived neutrophils. FACS derived dot plots represent apoptosis staining (annexin v) and necrosis, staining with propidium iodine (PI). Red squares highlight the percentage of neutrophils positive for apoptosis and necrosis.

B - JP2 survival after incubation with neutrophils treated with Nupharidine or vehicle**.** HL-60 derived neutrophils were incubated with JP2 (MOI 10) and with Nupharidine (12.5μg/ml or 25 μg/ml) or vehicle. Following incubation, supernatants were seeded on blood agar plate for CFU evaluation.

* indicates statistical significance (P≤0.05)

Fig 2 – Nupharidine effect on neutrophils Phagocytosis

HL-60 derived Neutrophils were incubated with FITC labeled JP2 (MOI 10) with Nupharidine (12.5µg/ml) or vehicle.

A – Cells underwent fixation and permeabilization, and then staining for phagosomes (LAMP1), and FITC-labeled JP2 bacteria and nuclei (DAPI). Microscopic images were taken at x4000 magnification.

B- Phagocytosis after Nupharidine treatment measured by FACS as percentage of FITC positive JP2 bacteria.

C – Amount of JP2 per cell in neutrophils measured by FACS as FITC geometric mean.

* indicates statistical significance ($P \leq 0.05$)

Fig 3 – Nupharidine effect on ROS in neutrophils

HL-60-derived neutrophils were incubated with unlabeled JP2 (MOI 10) with Nupharidine (12.5µg/ml) or vehicle. ROS was measured by 7'-dichlorofluorescein diacetate assay as total values (using florescent plate reader, or as internal ROS using flow cytometry.

A - Total ROS in the presence of JP2 with the presence on Nupharidine or vehicle.

B - Internal ROS in the presence of JP2 with the presence on Nupharidine or vehicle.

* Indicates statistical significance ($P \leq 0.05$).

Fig 4 – Nupharidine effect on NETosis

HL-60-derived neutrophils were incubated with unlabeled JP2 (MOI 10) with Nupharidine (12.5µg/ml) or vehicle. Membrane bound Extracellular DNA (NETosis) was quantified using MNase and SYTOX staining. Results are expressed as mean and SD of arbitrary florescent units (AFU).

* Indicates statistical significance (P≤0.05).

Fig 5 – The effect of Nupharidine on primary human neutrophils

Primary neutrophils were isolated from periodontally healthy and aggressive periodontitis subjects. Neutrophils were infected with JP2 (MOI 10) and in the presence of Nupharidine (12.5µg/ml) or vehicle.

A - JP2 survival (CFU) after Nupharidine treatment of neutrophils was determined**.**

B- Total ROS in the presence of JP2 with the presence on Nupharidine or vehicle.

* Indicates statistical significance (P≤0.05)

Fig 6 - Neutrophils priming markers following exposure to Nupharidine

HL-60 neutrophils were incubated with Nupharidine (12.5µg/ml) or vehicle. Following incubation, cells were stained for IL-1β production (internal staining) or iCAM and Dectin-2 (external staining). Analysis was done by FACS. Results are expressed as a histogram and compared with unstained cells (black histogram). Quantitative analysis of staining was done using geometric means values.

* indicates statistical significance (P≤0.05)