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HUMAN RANDOMIZED CONTROLLED TRIAL

Use of amnion-derived cellular cytokine solution for the treatment of gingivitis: A 2-week safety, dose-ranging, proof-of-principle randomized trial

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Abstract

Background: A 6-week Phase I clinical trial was performed to primarily evaluate the safety and secondarily determine the preliminary efficacy of a novel biological solution, ST266, comprised of a mixture of cytokines, growth factors, nucleic acids, and lipids secreted by cultured amnion-derived multipotent progenitor cells on gingival inflammation.

Methods: Fifty-four adults with gingivitis/periodontitis were randomly assigned to 1X ST266 or diluted 0.3X ST266 or saline topically applied on facial/lingual gingiva (20 μL/tooth). Safety was assessed through oral soft/hard tissue exam, adverse events, and routine laboratory tests. Efficacy was assessed by modified gingival index (MGI), bleeding on probing, plaque index, probing depth (PD), and clinical attachment level (CAL). Assessments were performed on day 0, 8, 12, and 42. ST266 and saline applied daily starting at day 0 through day 12 except weekend days. Plasma was analyzed for safety and proinflammatory cytokines, interleukin (IL)-1β, IL-6, tumor necrosis factor-alpha, and interferon gamma. Gingival crevicular fluid (GCF) was analyzed for the same cytokines. Subgingival plaque was primarily analyzed by checkerboard DNA-DNA hybridization. Comparisons with saline were modeled through a generalized estimating equations method adjusting for baseline.

Results: No safety concern was found related to ST266. Statistically significant reduction in MGI was noted at day 42 by 1X ST266 compared with saline ($P = 0.044$). PD and CAL were reduced by both doses of ST266 at day 42 ($P < 0.01$) and by 1X ST266 at day 12 ($P < 0.05$). GCF IL-1β and IL-6 levels were reduced by both doses of ST266 at day 12 ($P < 0.05$, $P < 0.01$, respectively). IL-6 was also

significantly reduced in plasma of both ST266 groups (P <0.05). Significant reductions in red complex bacteria were detected in both ST266 doses.

Conclusions: In this "first in human oral cavity" study, topical ST266 was safe and effective in reducing gingival inflammation in 6 weeks. Longitudinal studies with large sample sizes are warranted to assess the therapeutic value of this novel host modulatory compound in the treatment of periodontal diseases.

KEYWORDS

cytokines, gingival crevicular fluid, gingivitis, host modulation, inflammation, periodontal disease

1 | INTRODUCTION

Periodontal disease affects millions of Americans and adequate, long-term, stable therapy remains a clinical problem with an economic burden.[1] In periodontitis, an acute inflammatory reaction to commensal bacteria leads to inflammation and dysbiosis of the local microbiome.[2] If acute inflammation is not resolved and becomes chronic, there is innate and acquired immune pathway-mediated tissue destruction that leads to tooth loss.[3] Although infection by Gram-negative bacteria such as *Porphyromonas gingivalis* is considered to be the primary etiologic factor in periodontal disease, the cause-and-effect relationship between the pathogen and disease is not clear.[4] What is clear, however, is that a dysbiotic biofilm develops and if left unchecked, the periodontium including periodontal ligament and bone are destroyed. Mechanical debridement for biofilm removal has long been the standard prevention and treatment for gingivitis and periodontitis.[5] However, plaque removal is a short-term method for reducing inflammation; levels of biofilm bacteria rebound even in higher amounts after mechanical therapy in the absence of further treatment or maintenance which leads to a rapid return of the disease-associated hyper-inflammation.[6] Despite there being anti-bacterial and anti-plaque agents, only a few drugs/compounds are available to target the inflammatory response directly without significant side effects. To reduce the inflammatory response in tissues, drugs such as non-steroidal inflammatory agents have been used with limited clinical benefits and with a significant risk of unwanted side effects.[7] Similarly, systemic or local antibiotic therapy may provide a short-term benefit without a long-term solution and their use raises significant concerns due to increasing risk of antibiotic resistance.[8] New agents that stimulate the active resolution of inflammation may offer some therapeutic advantage in the treatment of periodontitis compared with more traditional pharmacologic interventions.[9]

Amnion-derived multipotent progenitor (AMP) cells produced by proprietary culturing of amnion epithelial cells obtained from non-controversial, donated full-term placentas, secrete a unique cocktail of cytokines at physiological levels.[10] The cells are non-tumorigenic and non-immunogenic, unlike other stem cells.[11] The cytokine secretome, referred to as ST266 (previously termed amnion-derived cellular cytokine solution or ACCS), tested in a variety of animal models,[12–15] accelerates and improves wound healing in both acute and chronic infected wounds regardless of change in bacterial bioburden.[13,16] In addition, ST266 is effective in the prevention and treatment of periodontitis in animal models.[17,18] In rabbit periodontitis, ST266 reduces inflammation and promotes healing and tissue regeneration.[18] In acute and chronic wound models,[13] skin grafting in rats[14] and in diabetic wound model in pigs,[12] it improves healing by accelerating wound closure and epithelization kinetics, promoting migration of keratinocytes and fibroblasts and increasing number of epidermal cell layers and rete ridges. ST266 promotes macrophage activity including increased phagocytosis and bactericidal activity.[19] Recently, treatment with ST266 ameliorated neuroinflammation in a rat model of brain injury and resulted in retinal ganglion cell neuroprotection in experimental optic neuropathies.[20,21] In a Phase I safety trial, topical application of ST266 in patients receiving breast radiotherapy was found safe in both intact and denuded, irradiated skin without systemic absorption.[22] In a Phase II clinical trial, topical ST266 was shown to reduce the acute effects of UV light-induced skin damage while reducing erythema and increasing DNA repair protein with decreased damaged DNA.[23]

Based on these safety data and the previous in vitro and in vivo findings in rabbit periodontitis, a Phase I randomized controlled clinical trial was designed to assess the safety and the preliminary efficacy of intra-oral topical application of two doses of ST266 in the reduction of gingival inflammation in patients with existing gingivitis/periodontitis.

2 | MATERIALS AND METHODS

2.1 | Ethical considerations and subject population

The study protocol was approved by the Forsyth Institute Institutional Review Board before initiation (Protocol #14-01) and conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. All subjects provided written informed consent before screening and enrollment procedures. The study was registered at ClinicalTrials.gov (NCT02071199).

Participants were recruited at the Center for Clinical and Translational Research (CCTR) clinic at the Forsyth Institute in Cambridge, Massachusetts. Individuals with good general health who consented to take part in the clinical trial, with an age range of 18 to 70 years, having at least 20 natural teeth excluding third molars, a whole mouth mean MGI score of ≥2 and at least 40% bleeding sites at initial presentation were enrolled in the study between April 2014 and June 2015.

Those with current medical conditions or on medications known to affect periodontal tissues or interfere with any of the study outcomes and those with history of cancer or any chronic infectious diseases were excluded. In addition, individuals with orthodontic appliances, pregnant and nursing women, and current or former smokers within 1 year of enrollment were excluded. Eligible subjects who met the inclusion/exclusion criteria were enrolled.

2.2 | Study design

This Phase 1 study was designed as a dose-escalating, randomized, prospective, double-blind parallel group clinical study to primarily test the safety and, secondarily, preliminary clinical efficacy of two concentrations of ST266 on gingivitis compared with saline as control. Study participants (n = 27) assigned to the first cohort received 0.3X ST266 (n = 18) or saline (n = 9) in a 2:1 (ST266:saline) randomization scheme. Study medication (20 μL/tooth, <1 mL/day) was topically applied to each tooth at the gingival margin by a calibrated pipette. The dose was chosen based on the preclinical animal data, filed in the original submission of the Investigational New Drug (IND) application, which demonstrated clinically significant difference compared with placebo in experimental periodontitis.[18] The frequency (daily) and duration of the treatment (2 weeks-total of 10 treatments) was based on the previous reports on topical use of ST266 in wound healing where bacterial bioburden was taken into consideration[14,16] After completion of treatment in the first cohort and upon receipt of a satisfactory unmasked safety review by the Data and Safety Monitoring Board, the separate second cohort (n = 27) received 1X ST266 (n = 18) or saline (n = 9) in the same randomization scheme of 2:1 (ST266:saline) (See Table S1 in online *Journal of Periodontology*). The subjects received 10 consecutive daily treatments over 2 weeks (except the weekend days), administered at Forsyth by a periodontist (HH) masked to the randomization scheme. Clinical assessments included safety and efficacy measurements performed at baseline, on day 8, at the end of the treatment phase (day 12) and at day 42 after the last treatment. An oral hygiene questionnaire was used to obtain current oral hygiene habits at baseline. All participants were given instructions for twice daily brushing with their regular manual toothbrush and a fluoride toothpaste, and not to change to another brand during the study. They were also instructed to stop using mouthwashes and chewing gums during the course of the study. Participants were provided a full-mouth professional tooth cleaning at the end of the study and referred to further periodontal treatment if deemed necessary.

2.3 | Primary outcome measure

Safety was the primary outcome measure and was monitored by evaluation of all adverse event/severe adverse event (AE/SAE) reports, subjects medical/medication history, concomitant medication reports, subject conduct/compliance, soft and hard tissue evaluations at every visit (daily) and dropout rates. Serum chemistry including C-reactive protein (CRP), liver and kidney function tests, complete blood count, urinalysis and, in women of childbearing potential, a pregnancy test was performed before enrollment and repeated upon completion of treatment on day 12.

To detect any adverse shifts in the supragingival microflora, plaque samples from six teeth (Ramfjord teeth) were collected, pooled and analyzed using DNA-DNA (checkerboard) hybridization[24] to provide a qualitative and semi-quantitative assessment of 13 oral bacteria, including *Fusobacterium nucleatum ss.vincentii, Campylobacter concisus, Campylobacter rectus, Bacteriodes forsythus, Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens, Capnocytophaga sputigena, Streptococcus oralis, Actinomyces naeslundii, Treponema denticola, Campylobacter curva, and Eikenella corrodens*. Results were compared with known probe standards and scored on an ordinal scale of 0 to 5 as previously reported.[25]

To determine the systemic exposure of locally delivered ST266, peripheral blood samples were collected via venipuncture before and 1 h after the first topical treatment

at day 0 and after the final treatment session on day 12. Plasma was separated, aliquoted, and stored at -80°C until analysis. Plasma levels of nine proteins known to be in ST266 were measured for the following analytes: platelet-derived growth factor, vascular endothelial growth factor, angiogenin, tissue inhibitor of matrix metalloprotease-1 (TIMP-1) and 2 (TIMP-2), carcinoembryonic antigen-125 (CA-125), decorin, matrix metalloprotease-9, and epidermal growth factor. Plasma samples were prepared and diluted according to the manufacturer's recommendations and run using a multiarray next generation of ELISA platform[*] and read on an image analyzer[†]. The concentrations were determined by comparison with a standard curve of recombinant protein. One of the aliquots of plasma was stored for proinflammatory cytokine analysis (a secondary outcome measure) at the Forsyth Institute Luminex Core as described below.

2.4 | Secondary outcome measures

2.4.1 | Clinical periodontal measurements

Preliminary efficacy was evaluated at baseline and day 8, 12, and 42 using multiple parameters, including modified gingival index (MGI)[26] at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual, and mesiolingual). Bleeding on probing (BOP) assessed as a dichotomous score of 0 or 1 after probing measurements were performed independently of the gingival index measurement and considered as the secondary gingivitis outcome variable. Amount of plaque on tooth surfaces was assessed using the Turesky Modification of Quigley-Hein Plaque Index (PI)[27] at six sites of the tooth. Probing depth (PD) and clinical attachment level (CAL) were measured using a UNC-15 periodontal probe[‡] at six sites per tooth.

2.5 | Examiner calibration

A single examiner (MM) masked to treatment allocations and not involved in topical treatments was responsible for all clinical oral measurements in a given subject for the course of the study. An intra-examiner calibration exercise was performed as previously described[28] with a minimum κ coefficient of 0.8.

2.6 | Proinflammatory cytokines

Changes in proinflammatory cytokines in the GCF and blood plasma were determined as an exploratory outcome measure. Gingival crevicular fluid (GCF) and peripheral blood plasma samples were obtained during the study to determine the changes in local and systemic levels of proinflammatory cytokines, respectively. GCF samples were collected at day 0, day 12, and day 42 from the most inflamed mesiobuccal sites of four teeth, one per quadrant based on MGI score (highest MGI score), using sterile PerioPaper strips[§] for 30 seconds. Following collection, the samples were snap-frozen in liquid nitrogen and stored at -80°C until analysis.

On the day of analysis, the frozen GCF samples were thawed at room temperature and proteins were eluted through two centrifugations at 13,000 × g at 4°C for 8 minutes in a total of 120-µL sterile phosphate buffered saline (PBS, pH 7.4). In all analyses, 100 µL of eluted solution was used. In parallel, plasma samples were also thawed at room temperature and 1:2 dilutions prepared. Both the GCF and plasma samples were analyzed for proinflammatory cytokines including interleukin (IL)-1β, IL-6, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) using a 4 plex human high sensitivity cytokine panel[¶] by multiplexed sandwich immunoassays, based on flowmetric multiplex technology[#] at the Forsyth Institute Luminex Core as described previously.[29] Briefly, assays were carried out on a flowmetric multiplex machine[†] and data were read using the image reader.[**] Immediately before the initiation of study measurements, the multiplex platform underwent a complete on-site maintenance cycle and operationally qualified by the manufacturer's field engineers. Daily and weekly performance qualification was continuously verified by the Core staff during the analytical period. Assay analysis was performed according to manufacturer's protocols. Single lot numbers of each kit were used to minimize analytical variability. Reagents provided in these kits included magnetic beads pre-coated with capture antibodies, standards, assay diluents, biotin-conjugated secondary antibodies, biotin diluent, streptavidin conjugated to the fluorescent protein, R-phycoerythrin (streptavidin-RPE), streptavidin-RPE diluent, washing buffer concentrates, and assay buffers, as well as the 96-well filter or magnetic plates. The quality control measurements and coefficient variations were calculated as described previously by the same laboratory.[29] The detection limits of the

[*] Meso Scale Discovery, Rockville, MD
[†] Meso Scale Discovery Model 1200 Sector Imager 2400, San Diego, CA
[‡] Hu-Friedy, Chicago IL
[§] Oraflow, Smithtown, NY
[¶] Luminex Human High Sensitivity Cytokine Magnetic Panel B, R&D Systems, Minneapolis, MN
[#] Luminex 100 Bio-Plex Platform, Bio-Rad Laboratories, Hercules, CA
[**] Bio-Plex Manager version 6.1, Bio-Rad Laboratories, Hercules, CA

kits were 0.146 pg/mL for IL-1β, 0.14 pg/mL for IL-6, 0.29 pg/mL for TNF-α, and 0.25 pg/mL for IFN-γ.

2.7 | Investigational product

ST266 is a complex mixture of proteins, including cytokines and growth factors, nucleic acids, and lipids produced by cultured AMP cells. ST266 final drug product, lot number, E2751301, was aseptically filled and packaged[††] from bulk drug substance produced at Good Manufacturing Procedures facilities.[‡‡] Saline was used as the placebo.

2.8 | Treatment compliance and data quality

Treatment applications were performed in the dental clinic by the study investigator; therefore, non-compliance was assessed based on the treatment visits missed. Data quality was monitored during monitoring visits by an external clinical research associate at regular intervals for the duration of the trial.

2.9 | Statistical analysis

2.9.1 | Power analysis

As the primary efficacy measure, modified gingival index was used to calculate sample size. Based on published studies, a sample size of 18 subjects per group (assuming a dropout rate of 20%) was calculated, using a generalized estimating equations (GEE) model for repeated measurements with an SD of 0.3 MGI units, 0.05 significance level; power of 80%; and exchangeable correlation structure with a correlation coefficient of $\rho = 0.3$ among repeated measurements. Fourteen (14) subjects in each group were needed to detect a difference of 0.2 units (mean MGI change). To compensate for attrition, assuming a 20% drop rate, 18 subjects per treatment group were enrolled in the study.

2.9.2 | Statistics

Safety parameters compared across treatments (1X ST266 versus placebo and 0.3X ST266 versus placebo) included adverse events, vital signs, and oral cavity examination using GEE modeling. The fit of the linear regression models was done by using a GEE approach, estimating the temporal trend in each vital sign over the sequence of visits, by group. A fit of interaction term (visit*treatment) was done for each of the two dose groups. The temporal trend is an estimate of the average per day change in the vital sign. The interactions allow for estimation and testing of whether the temporal trend (rate of change) over the visits is different by treatment group. Other safety end points included blood chemistry, hematology, urinalysis, and levels of CRP before treatment (day 0) and on day 12. Tests on day 12 were repeated if any out of value was deemed clinically significant as confirmed by the medical monitor. For each of the four (4) end points, the number of values out of the normal range (0, 1, 2, 3, 4, >4) at each time point was recorded. The repeat day 12 report was used if performed. The number and frequency of subjects with each number of abnormal values for each analysis and both time points were tabulated.

To detect any adverse shifts in the supragingival microflora, plaque samples from six teeth were collected pooled and analyzed using DNA-DNA hybridization checkerboard. Values were then $\log_{10}$-transformed. We used linear regression with robust standard errors to compare mean differences between each treatment group versus the placebo group.

Analysis of the efficacy data consisted of modeling end points, including MGI, BOP, PD, CAL, and PI with groups as the main effect adjusted for baseline. Treatment comparisons at post baseline were modeled through GEE (with a linear link, exchangeable covariance matrix, and robust variance) adjusting for the baseline value. GEE is an approach that accounts for repeated measures (i.e., multiple sites within each subject's mouth). Proinflammatory cytokine levels in GCF and in plasma were also analyzed using GEE modeling.

In secondary analyses, the rate of change over time was estimated with GEE analyses. The model included time (in days), treatment group indicators (with placebo as the reference group), and multiplicative interactions of each treatment indicator multiplied by time. The average rate of change per day is reported in each treatment group, and the differences in these rates of change for each treatment group (versus placebo). In the case of missing visits, the data were computed based on the number of treatments the subject received (minimum three treatment visits out of five completed at each week). All analyses were performed using a commercially available statistical package.[§§]

[††] Afton Scientific, Charlottesville, VA
[‡‡] Noveome Biotherapeutics, Pittsburgh, PA

[§§] Stata version 13.0; College Station, TX

3 | RESULTS

3.1 | Study participants

Out of 196 subjects screened according to inclusion and exclusion criteria of the study, 63 subjects were found to be eligible and scheduled for a baseline visit. Five subjects dropped out before the baseline visit (four withdrew consent because of unavailability and one was lost to follow-up). Of 58 subjects who came for the baseline visit, four subjects were terminated at baseline before randomization and treatment; one subject withdrew consent, one was non-compliant, and two subjects were ineligible based on the inclusion criterion (<40% BOP) (see Figure S1 in online *Journal of Periodontology*). The remaining 54 subjects randomized into one of the study treatments completed the entire study participation and the safety and efficacy data obtained were included in the data analyses. All participants responded to questionnaire with at least once daily brushing, flossing at least 3 times/week, 21% were using mouthwashes and none were using irrigation devices (data not shown). During the study all participants continued using their regular manual toothbrush and a fluoride toothpaste twice a day and discontinued using mouthwashes. Approximately 33% (n = 18; 7 in placebo, 6 in 0.3x ST266; 5 in 1x ST266) of study population presented with localized periodontitis with PD 5 to 9 mm at ≤4 interproximal sites (see Table S2 in online *Journal of Periodontology*).

3.2 | ST266 is safe, well tolerated, and reduces red-complex periodontal pathogens

3.2.1 | Adverse events (AEs)

There were 124 reported events and oral cavity exam findings. All 124 events were categorized as mild; 3 (2.4%) were categorized as related, 5 (4%) were categorized as possibly related, and 5 (4%) were categorized as unlikely related (see Table S3 in online *Journal of Periodontology*). All related and possibly related events were reported by the same two subjects who were assigned to placebo. All events were resolved regardless of study group. There were no AEs related to drug and no pattern of concern in the AEs reported. There were no serious AEs.

3.2.2 | Vital signs

For all vital sign safety end points, except temperature, there were relatively few readings outside the normal range and no lasting pattern by treatment group or over time (see Table S4 in online *Journal of Periodontology*). The temperatures outside the normal range were generally low and could have been affected by fluid intake before oral measurement.

Although there were minor differences in some of the vital sign measures by treatment group on day 1, there was no evidence that over the course of treatment the temporal trends in vital signs were different by treatment group.

3.2.3 | Microbial analysis

Microbial analysis conducted primarily for safety of topical ST266 application showed no adverse shifts (increases in pathogenic bacteria) in the microflora (see Table S5 in online *Journal of Periodontology*). On the contrary, the bacterial counts at day 10 showed reductions in red and orange complex bacteria known to be highly associated with periodontal disease (e.g., increased PD and BOP) compared with baseline counts in both ST266-treated groups (Fig. 2 and see Figure S2 in online *Journal of Periodontology*).

3.2.4 | Measurement of ST266 cytokines in plasma

Baseline protein levels varied slightly among groups. Local administration of ST266 in either dose did not increase protein levels measured 1 hour after the first or tenth dose of ST266 application.

3.3 | ST266 reduces gingival inflammation and local proinflammatory cytokines

3.3.1 | Clinical periodontal outcomes

Gingival index, BOP, PD, and CAL decreased over time with the treatment of both doses of ST266 at all time-points; day 8, 12, and 42 and changes from baseline were greater compared with placebo (analysis adjusted for baseline differences) (Table 1). The change in primary end point MGI at day 42 with 1X ST266 reached statistical significance when compared with placebo (difference 0.1; $P = 0.044$; Fig. 1). Change in BOP approached statistical significance at day 8 with both doses of ST266 compared with placebo ($P = 0.05$, and $P = 0.06$, respectively). In formal analyses of mediation, this change in modified gingival index accounted for a modest but statistically significant proportion of the effect of ST266 treatment on the

TABLE 1 Clinical outcomes: gingival index, bleeding on probing, and plaque index

Time point and comparison	Mean gingival index (MGI unit)				Bleeding on probing (% of sites)				Plaque index (PI unit)			
	Mean diff.	95% CI Lower	Upper	*P* value	Mean diff.	95% CI Lower	Upper	*P* value	Mean diff.	95% CI Lower	Upper	*P* value
8 days												
0.3X ST266 minus placebo	−0.04	−0.13	0.05	0.434	−9.04	−0.18	0.00	0.051	−0.13	−0.32	0.07	0.193
1X ACCS minus placebo	−0.06	−0.14	0.02	0.121	−8.68	−0.18	0.00	0.062	−0.05	−0.23	0.14	0.641
12 days												
0.3X ST266 minus placebo	−0.04	−0.13	0.05	0.394	−6.24	−0.16	0.04	0.226	−0.12	−0.33	0.09	0.263
1X ACCS minus placebo	−0.03	−0.13	0.07	0.519	−5.24	−0.15	0.05	0.311	−0.02	−0.21	0.17	0.819
42 days												
0.3X ACCS minus placebo	−0.03	−0.12	0.06	0.528	−4.81	−0.12	0.03	0.219	0.11	−0.13	0.35	0.383
1X ACCS minus placebo	−0.10	−0.20	0.00	0.044*	−3.37	−0.11	0.04	0.392	−0.04	−0.29	0.21	0.760

*Significant difference $P < 0.05$. Mean clinical differences, 95% confidence intervals (CI), and *P* values (not multiple-comparison corrected) comparing 0.3X ST266 and 1X ST266 to placebo using GEE analysis (adjusted for baseline).

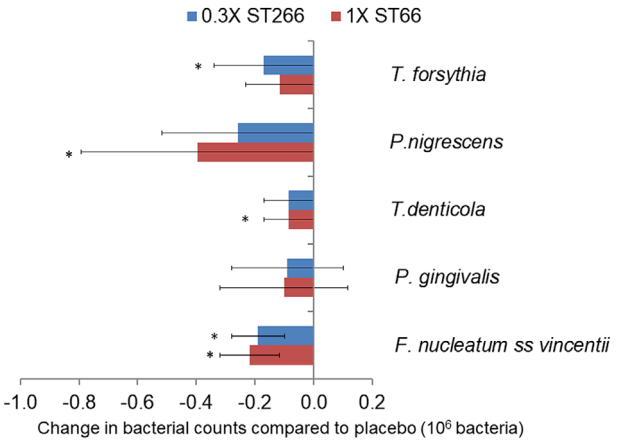

FIGURE 1 Change in primary outcome, gingival index, at day 42. Differences were detected in gingival index (MGI) between placebo and both doses of ST266 at all time points; day 8, 12, and 42 (analysis adjusted for baseline differences). Statistically significant differences were noted for primary efficacy end point MGI at day 42 with 1X ST266 compared with placebo. *Significant difference at $P < 0.05$

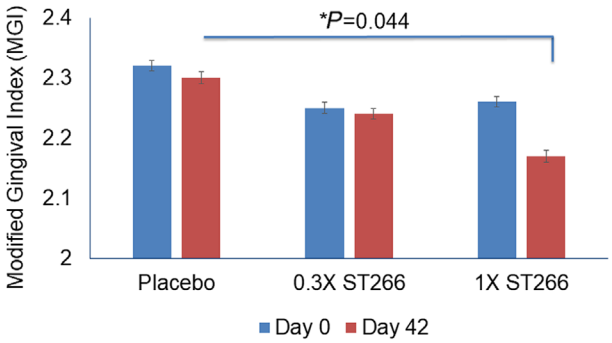

FIGURE 2 Change in bacterial counts of periodontal species. Fourteen periodontal bacteria were semi-quantitively analyzed using DNA-DNA hybridization checkerboard assay at baseline and at day 10 after treatments. Mean differences of change from baseline in ST266-treated groups were compared with the change from baseline in place group. Both red and orange complex species known to be strongly associated with periodontal disease showed significant reductions on day 10 after treatment with ST266. Mean differences, 95% confidence intervals, and *P* values comparing 0.3X ST266 and 1X ST266 to placebo using GEE analysis. *Significant difference at $P < 0.05$

likelihood of BOP reduction calculated as odds of bleeding (mean decrease by the treatment: 14%; 95% confidence interval, 3%–35%; Table 2). No statistically significant differences were found in PI at any time point (Table 1).

Reductions in exploratory efficacy end points, PD and CAL, were detected at all time-points with 0.3X ST266 and 1X ST266 at day 42 (PD, $P = 0.005$ and $P = 0.008$; and CAL, $P = 0.002$ and $P = 0.001$, respectively) compared with placebo (analysis adjusted for baseline differences) (Table 3). On day 12, there were changes for both PD ($P = 0.03$) and CAL ($P = 0.014$) in 1X ST266-treated group compared with placebo. The changes from baseline were statistically significantly different when compared with placebo for both PD and CAL.

The average daily change in PD, CAL, and MGI was calculated over time as rate of change. Placebo group showed increases in PD and CAL while both treatment groups (1X and 0.3XST266) had decreases in both PD and CAL over time ($p_{0.3X} = 0.07$, $p_{1X} = 0.032$) and CAL ($P < 0.0001$ for both groups compared with placebo). In addition, 1X ST266 showed significantly greater average change in MGI compared with placebo ($P = 0.044$) (see Table S6 in online *Journal of Periodontology*).

Finally, the change in PD was also analyzed by stratifying the data according to PD. Both ST266 treatment groups showed statistically significant reductions at day 42 in shallow pockets (1 to 3 mm) and deeper pockets

TABLE 2 Odds of bleeding compared with placebo

Time point and comparison	Bleeding on probing (yes/no each site) Mean difference	95% CI Lower	Upper	*P* value
8 days				
0.3X ACCS minus placebo	−0.35	−0.88	0.18	0.197
1X ACCS minus placebo	−0.75	−1.25	−0.24	0.004*
12 days				
0.3X ACCS minus placebo	−0.25	−0.89	0.40	0.452
1X ACCS minus placebo	−0.73	−1.38	−0.07	0.030*
42 days				
0.3X ACCS minus placebo	−0.22	−0.70	0.25	0.355
1X ACCS minus placebo	−0.40	−0.84	0.04	0.071

*Significant $P < 0.05$; GEE/logistic model, repeated measures for each subject and outcome measured as 0/1 for no/yes.

TABLE 3 Exploratory clinical outcomes: probing depth and clinical attachment level

Time point and comparison	Probing depth Mean difference	95% CI Lower	Upper	*P* value	Clinical attachment level Mean difference	95% CI Lower	Upper	*P* value
8 days								
0.3X ACCS minus placebo	−0.041	−0.132	0.050	0.374	−0.050	−0.156	0.056	0.353
1X ACCS minus placebo	−0.027	−0.116	0.062	0.556	−0.075	−0.174	0.024	0.137
12 days								
0.3X ACCS minus placebo	−0.103	−0.220	0.015	0.087	−0.072	−0.185	0.042	0.217
1X ACCS minus placebo	−0.129	−0.245	−0.013	0.030*	−0.136	−0.244	−0.027	0.014*
42 days								
0.3X ACCS minus placebo	−0.134	−0.228	−0.040	0.005*	−0.173	−0.285	−0.062	0.002*
1X ACCS minus placebo	−0.134	−0.234	−0.035	0.008*	−0.199	−0.314	−0.084	0.001*

*Significant difference $P < 0.05$; mean clinical differences, 95% confidence intervals (CI), and *P* values (not multiple-comparison corrected) comparing 0.3X ST266 and 1X ST266 to placebo using GEE analysis (adjusted for baseline).

(4 to 9 mm) compared with placebo group ($p_{0.3X} = 0.022$, $p_{1X} = 0.037$ and $p_{0.3X} = 0.000$, $p_{1X} = 0.003$, respectively; data not shown).

The overall clinical periodontal measurement data by treatment group and by time point including baseline levels can be found in Table S7 in online *Journal of Periodontology*.

3.3.2 | Local and systemic inflammatory mediators

Treatment with both doses of ST266 resulted in significant reduction in GCF levels of IL-1β and IL-6 compared with placebo at day 12 after the tenth treatment (IL-1β, $P = 0.02$, $P = 0.015$; and IL-6, $P = 0.005$, $P = 0.002$, respectively) (Fig. 3). Only small differences were observed in the GCF levels of TNF-α and IFN-γ was undetectable in plasma and at very low or undetectable levels in the GCF.

Plasma levels of IL-6 was significantly reduced compared with placebo in both 0.3 X ST266 and 1X ST266 groups 1 hour after the first treatment ($P = 0.019$ and $P = 0.03$, respectively) (see Table S8 in online *Journal of Periodontology*).

4 | DISCUSSION

This was a "first in human" oral topical application of ST266 primarily for safety and preliminary efficacy on gingival inflammation. Subjects with gingivitis and/or periodontitis exhibiting at least 40% of BOP and 2.0 MGI score on average were included in the study. The safety analyses including reported adverse events, oral and hard tissue findings, vital signs, and routine laboratory tests (hematology, blood chemistry, C-reactive protein, and urinalysis) showed no adverse outcomes related to study product. Reported adverse events by subjects in the ST266

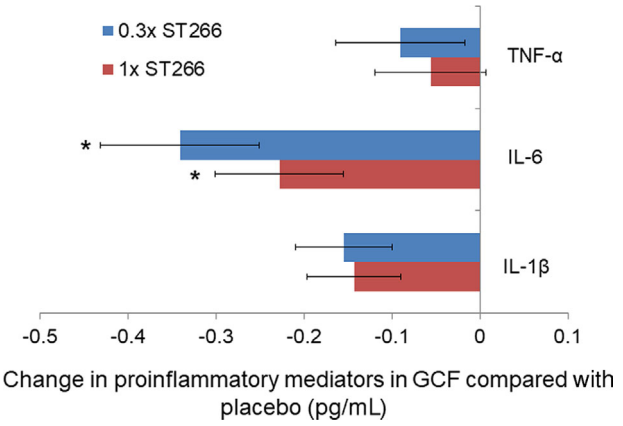

FIGURE 3 Change in levels of proinflammatory cytokines in gingival crevicular fluid. Levels of proinflammatory cytokines including IL-1β, IL-6, and TNF-α in GCF were determined using a multiplexing platform. Mean differences of change from baseline in ST266-treated groups were compared with the change from baseline in place group. Significant reductions were found in the levels of IL-1β and IL-6 in the GCF of subjects assigned to both 0.3X ST266 and 1X ST266 compared with placebo at day 12 after the tenth treatment (IL-1β, $P = 0.02$, $P = 0.015$; and IL-6, $P = 0.005$, $P = 0.002$, respectively). Mean differences, 95% confidence intervals, and P values comparing 0.3X ST266 and 1X ST266 to placebo using GEE analysis. *Significant difference at $P < 0.05$

groups were either unlikely related or not related. Minor differences were detected in some of the vital sign measures by treatment group at baseline; however, there was no evidence that over the course of treatment the temporal trends in vital signs were different by treatment group. Oral microbial analysis was conducted primarily for safety, which showed no adverse shift towards pathogenic microflora; on the contrary, both ST266 groups showed reduced levels of key red complex bacteria (*Porphyromonas gingivalis, Tannerella forsythia, and Treponema denticola*) reported to be highly associated with periodontal disease.[4,30]

As one of the main goals of periodontal treatment is reducing the inflammation, several anti-inflammatory agents have been considered for use as adjunctive treatments including corticosteroids, disease modifying anti-rheumatic drugs and non-steroidal anti-inflammatory drugs.[7,31] The studies evaluating the possible effects of corticosteroids and disease modifying anti-rheumatic drugs on periodontal and gingival inflammation have been performed among the patients who were taking these drugs for other indications. Both drugs were associated with a range of side effects and did not have significant effects on reducing the inflammation in gingival tissues.[7,31]

On the other hand, non-steroidal anti-inflammatory drugs have been shown to reduce periodontal disease progression, especially in long-term uses.[7,32] The most common finding of the studies was reduced alveolar bone loss when compared with the patients who were not taking non-steroidal anti-inflammatory drugs. Patients who took non-steroidal anti-inflammatory drugs for prolonged periods also tend to have reduced gingival inflammation and reduced PDs.[33–35] However, a high rate of side effects were also associated with long-term use of non-steroidal anti-inflammatory drugs including gastrointestinal, renal and hemostatic side effects, hypersensitivity reactions, headaches, dizziness, and vertigo.[7] In our study, significantly reduced gingival and local inflammation as well as PD were observed with no adverse events related to any of the doses of ST266.

Recently, several studies have reported the potential use of probiotics for reducing gingival inflammation.[36–38] Although some individual studies lean towards the use of probiotics, in a meta-analysis in 2020 it was concluded that there is weak evidence to support the use of probiotics in gingivitis.[36] Here, we demonstrate improvement in most of the clinical parameters tested with the use of ST266 over 42 days. The primary preliminary endpoint, modified gingival index, was reduced at day 42 by 1X ST266 compared with placebo. Both 0.3X ST266 and 1X ST266 resulted in reductions in PD and clinical attachment loss at day 42 with an overall trend in reduction throughout the course of the treatment despite any mechanical treatment. The PD and CAL reductions were noticeable as early as day 12 with 1X ST266 group compared with placebo. Although, the study primarily tested the novel treatments on gingival inflammation in patients with gingivitis and therefore powered for detecting significant differences in gingival inflammation, the study protocol also included patients with periodontal disease exhibiting similar level of gingival inflammation. Reductions in both PD and attachment loss offer a promising benefit from ST266 for the treatment of periodontal diseases.

A combination of antibacterial and anti-inflammatory agents is considered as a viable option for the treatment of periodontal diseases.[9] For example, adjunctive use of systemic tetracycline and ibuprofen with scaling and root planing was shown to be beneficial when compared with scaling and root planing only.[9,39] A macrolide antibiotic, azithromycin, which has both antibacterial and anti-inflammatory properties,[40] was also reported to improve the efficacy of non-surgical periodontal therapy by reducing the PD, BOP, and increasing the gain of attachment.[9,41] The results of the studies using antibiotics as adjunctive periodontal therapies seems to have promising outcomes with regard to the clinical outcomes, however potential changes in the host microbiome, a small risk of adverse cardiovascular events and the risk of antibiotic resistance should be considered as a downside of these treatment alternatives.[9,42]

In the present study, parallel to reductions in gingival index and PD, inflammatory cytokines in the gingival crevicular fluid, IL-1β, and IL-6, were reduced at day 12 in response to both ST266 treatments. Concurrently, levels of subgingival periodontal microorganisms *F. nucleatum, T. denticola*, and *T. forsythia* showed reductions compared with placebo at day 12 in both the 0.3X or 1X ST266 groups suggesting that ST266 treatment can modulate the inflammatory response and subgingival microbiome shifting the balance from disease associated to health associated profiles. Studies designed to determine the impact of ST266 treatment on oral microbiome are warranted to further prove this hypothesis.

A relatively new approach is the administration of omega 3 polyunsaturated fatty acids (ω-3 PUFA) and acetylsalisylic acid (ASA) as adjuncts to periodontal debridement for the treatment of periodontitis to control inflammation.[43,44] In a study comparing the use of ω-3 PUFA and ASA for two different treatment protocols in patients with diabetes, reductions in IL-1β and IL-6 were reported, especially in patients who had periodontal debridement before ω-3 PUFA and ASA administration.[45] Similar to ω-3 PUFA and ASA, in our study ST266 reduced IL-1β and IL-6 levels by day 10 (in 12 days) and could be considered a novel therapeutic in patients with periodontal disease complicated by systemic inflammatory diseases including diabetes. The decreased levels of decorin, an inflammatory biomarker,[46,47] in plasma also indicates the anti-inflammatory actions of topical oral ST266 that warrants further investigation on the benefits of ST266 on systemic inflammation through oral tissues. The results of this Phase 1 clinical proof-of-principal study support the hypothesis that topical application of ST266 reduces periodontal inflammation at both clinical and biochemical levels in a short period (2 weeks) with stability at 6 weeks post-treatment. Reduced numbers of periodontal pathogens in ST266-treated sites also support the concept of host-modulation as an optimal therapeutic approach for this multifactorial disease.

As a Phase 1 proof-of-principle clinical trial primarily focusing on the safety, the study had small sample size and therefore limited power for detecting differences in clinical efficacy outcome measures. However, the study results, for the first time, demonstrated that ST266 could be a potentially impactful therapeutic for the treatment of periodontal disease with consistent improvements on the clinical (gingival inflammation) and biological end points (local cytokines and microbial species). Although there were close-to-significant reductions, this study likely underestimated the true impact on systemic proinflammatory cytokines and larger trials will be necessary to test the potential of ST266 as a topical treatment on the regulation of systemic inflammatory mediators. The relatively short duration of the treatment (10 consecutive days) should also be tested for long-term effects especially due to the chronic and progressive nature of periodontal disease. Further, plaque sampling from four posterior sites may not be representative of periodontally affected sites and the checkerboard analysis technique only examined a subset of the gingival microbiome. Yet, significant reductions have been shown with periodontal pathogens including *P. gingivalis* and *T. forsythia*.

5 | CONCLUSIONS

Topical ST266 applied directly on to marginal gingiva was safe and effective in reducing gingival inflammation in 6 weeks. With statistically significant reductions in BOP, PD, and CAL, ST266 shows promising therapeutic value as a novel host modulatory compound in the treatment of periodontal diseases. Longitudinal studies with large sample sizes and dose response designs are warranted to characterize its clinical efficacy and potential impact on inflammatory conditions related to periodontal diseases.

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All authors except Drs. Steed and Van Dyke report no conflicts of interest related to this study. Dr. Steed is the Executive VP of Medical Affairs at Noveome Biotherapeutics, owns stocks, and filed multiple patents on ST266. Dr. Van Dyke has been a paid advisor and owns stocks in Noveome Biotherapeutics.

AUTHOR CONTRIBUTIONS

Hatice Hasturk substantially contributed to conception and design, acquisition, analysis, and interpretation of data; drafted and critically revised the article for important intellectual content. David Steed contributed to conception and design, analysis and interpretation of the data, critically revised the article. Emre Tosun and Thomas E. Van Dyke critically revised the manuscript with important intellectual contribution. Melissa Martins and Constantinos Floros performed data and sample collection and data entry. Maryann Cugini supervised randomization, study execution and data acquisition. Daniel Nguyen and Danielle Stephens performed analysis of biological

specimens and critically revised the analytical sections. Jacqueline Starr performed statistical analyses and contributed to interpretation of the results. All authors approved the final version of the article and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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