Original Article

In vivo (human) and *in vitro* inactivation of SARS-CoV-2 with 0.5% povidone-iodine nasal spray

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Background: Nasal disinfection with 0.5% povidone-iodine (PVP-I) may be a useful adjunct in the management of COVID-19. The purpose of this article is to confirm the *in vitro* activity of the PVP-I nasal spray against SARS-CoV-2 and whether that may translate into reduced nasal shedding *in vivo*.

Methods: Two SARS-CoV-2 virus isolates were exposed to 0.5% PVP-nasal spray (Nasodine®) for different times *in vitro*, with PCR and cell culture used to assess impact on viral infectivity and RNA copies. An open label *in vivo* single arm pilot study of 14 subjects with positive COVID-19 PCR diagnosis was undertaken. Baseline nasal swabs were collected to quantify SARS-CoV-2 pre-treatment, followed by a single 0.5% PVP nasal spray application (1.12 mL). Nasal swabs were collected at 5, 15, and 60 minutes post-dose to assess immediate and residual impact of treatment.

Results: *In vitro*, the nasal spray reduced infectivity by 3.5 log10 $TCID_{50}$/mL (99.97%) after 15 seconds exposure and eliminated detectable viral infectivity after 60 seconds; there was no effect on viral RNA detection by PCR. *In vivo*, culturable virus (VOC beta/B.1.351 variant) was obtained from 6 of 14 PCR-confirmed positive subjects; in these subjects, 5 minutes after the single PVP-I dose, the mean viral titre was reduced by 65% versus baseline and by 79% versus baseline at 60 minutes post-dose. 5 of the 6 subjects (83%), had reduction or cessation of viral shedding at 5 minutes in all 6 subjects, virus titers 60 minutes post-dose were below baseline value. 0.5% PVP-I treatment didn't interfere with the laboratory diagnosis of COVID-19 via PCR-detection of viral RNA in humans.

Conclusions: 0.5% PVP-I nasal spray is rapidly virucidal to SARS-CoV-2 *in vitro* using exposure times consistent with nasal residence; single *in vivo* nasal administration reduced infectious viral titers in COVID-19 subjects with culturable virus. A single application of 0.5% PVP-I nasal spray does not interfere with PCR-mediated laboratory diagnosis of COVID-19. We are undertaking a large double blinded randomized controlled trial to confirm if repeated application of 0.5% PVP-I nasal spray over a longer period could be useful in suppressing viral shedding and transmission risk in COVID-positive patients.

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Introduction

Global management of the COVID-19 pandemic relies on effective community vaccination and preventive strategies to reduce airborne spread of the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) virus.

SARS-CoV-2, the virus responsible for COVID-19, replicates effectively in the upper respiratory tract (URT) with an apparent preference for the nasal passages, exhibiting a higher viral load and viral replication rate than the oral cavity and lower respiratory tract (LRT) (1,2). The virus is readily isolated from the nose early in the course of the disease, often prior to any symptoms, and shedding from the nose and URT is thought to be a primary mode of transmission (3). Therefore, the nasal cavity presents an important target for early disinfection and viral elimination, especially while vaccination is not universally available, and the virus continues to mutate with the potential to circumvent vaccination.

Povidone-iodine (PVP-I) is a microbicide that is rapidly active against all tested viruses at low concentrations (4-6). The use of PVP-I in the nasal passages has been proposed as an intervention to assist in the management of SARS-CoV-2 infection (7,8)—through localized disinfection of the nasal passages as a means of augmenting PPE and hand disinfection practices during the COVID-19 pandemic (9-13). The proposals are largely based on *in vitro* data and none of the proposed PVP-I formulations has been rigorously tested for safe and effective nasal use in human clinical trials.

However, for several reasons, *in vitro* data on the activity of PVP-I formulations against SARS-CoV-2 may not translate into *in vivo* safety and efficacy. Disinfection of the functioning nasal epithelium is complicated by rapid clearance of materials from the nasal cavity, due to mucociliary clearance and nasal discharges that can dilute and remove locally applied solutions, and in the case of PVP-I, mucins in nasal mucous may directly inactivate free iodine. From a safety perspective, high concentrations and/or volumes of PVP-I are unacceptable for nasal use due to ciliotoxicity, local sensitivity and the risk of iodine uptake through the nasal mucosa (14-16). Any intranasal formulation of PVP-I needs to be easily administered, stable for distribution and use, effective in the nose as a virucide despite short effective exposure times, and above all, safe for intranasal use.

Nasodine® Nasal Spray (Nasodine) is a commercial formulation of 0.5% PVP-I, manufactured under good manufacturing practice (GMP) and optimized for the safe and effective use on the nasal epithelium. Preclinically, it was tested in a sensitive air-liquid interface (HNEC-ALI) model of human nasal epithelium and even after 30 minutes exposure time, the formulation produced no ciliotoxicity, no detrimental effects on the paracellular permeability, and no indication of cellular toxicity (14). Subsequently, the product was developed as a treatment for the common cold (Nasodine® Nasal Spray is sponsored by Firebrick Pharma Ltd, Melbourne Australia) and assessed in human studies for safety and efficacy, including a Phase III randomized controlled trial in adults with cold symptoms (ANZCTR: ACTRN12619000764134). The product is currently under review for marketing approval as an over-the-counter medicine for treatment of the common cold.

Prior to the COVID-19 pandemic, Nasodine's activity was confirmed *in vitro* against representative strains of all major viruses responsible for respiratory infections. We present the following article in accordance with the TREND reporting checklist (available at https://ajo.amegroups.com/article/view/10.21037/ajo-21-40/rc).

The overall objective was to establish the activity of 0.5% PVP-I nasal spray against SARS-CoV-2 *in vitro* and then assess in a pilot human study whether and how that activity translated *in vivo* in confirmed COVID-positive adults. The pilot study was designed to provide preliminary evidence of an immediate and short-term sustained impact of a single application of Nasodine to the nasal passages of COVID-positive patients and, if warranted, to guide the design of future studies. A secondary objective was to assess whether there was an effect on PCR-detectable virus after exposure to the formulation, in order to determine if use of the nasal spray could interfere with concurrent or subsequent COVID-19 diagnosis.

Methods

The trial was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Sir Charles Gairdner and Osborne Park Health Care Group Human Research Ethics Committee (EC 00271/RGS0000004334) and South African Pharma-Ethics (200923548) and South African Health Products Regulatory Authority (20200439) and informed consent was taken from all individual participants.

In vitro studies

Nasodine Nasal Spray (0.5% povidone-iodine) was supplied by Firebrick Pharma Limited, Melbourne Australia. Two *in vitro* studies were conducted: (I) a preliminary study in 2020 at the Victorian Infectious Diseases Reference Laboratory (VIDRL), using an early isolate of SARS-CoV-2 and a 60-second Nasodine exposure time; and (II) a confirmatory *in vitro* study conducted at Utah State University, with a later isolate, more established culture and detection methods, and multiple Nasodine exposure times from 15 seconds to 15 minutes.

The preliminary study used BetaCoV/Australia/VIC01/2020 isolated and grown by VIDRL in January 2020 from a positive patient specimen. The virus (at 1.2×10^5 $TCID_{50}$ units/mL) was incubated in Eagle's Minimum Essential Medium (EMEM) alone (negative control) or Nasodine at 37 ℃ for 1 minute; the reactions were then stopped by 10-fold dilution using ice cold medium containing 2% foetal bovine serum (FBS) to inactivate any residual PVP-I prior to culture. Since cytopathic effects (CPE) associated with BetaCoV/Australia/VIC01/2020 were uncertain at the time of study, to confirm the presence or absence of replicating SARS-CoV-2, samples from all culture plate wells were analyzed for the presence of SARS-CoV-2 RNA by real-time RT-PCR, rather than based on CPE.

The second study used SARS-CoV-2/USA_WA1/2020, which was prepared by Natalie Thornburg, CDC and provided by WRCEVA, University of Texas Medical Branch. Virus stocks were prepared in Vero E6 cells. Nasodine was compared with a virus control solution, incubated at 37 ℃ for 15 seconds, 5 minutes, and 15 minutes, before neutralization by a 1/10 dilution in test media containing 10% FBS and then assayed by TCID assay using 8 log_{10} dilutions in test medium. Each dilution was added to 4 wells of a 96-well plate with 80–100% confluent Vero E6 cells and incubated at 37 ℃, 5% CO_2. On day 6 post-infection, plates were scored for presence or absence of viral cytopathic effect (CPE), with the Reed-Muench method used to determine end-point titers ($TCID_{50}$) and the log reduction value (LRV) of Nasodine compared to the control.

In vivo study

A pilot viral shedding study in COVID-positive patients was conducted at Jongaie Research in Pretoria, South Africa, between October and December 2020 (ANZCTR: ACTRN12620000470998) and (SANCTR: DOH-27-122020-6373). The study was a single-arm, uncontrolled, un-randomized, open-label study.

The intention to treat (ITT) population was 14 laboratory-confirmed (PCR), COVID-19 positive subjects with recent COVID-19 symptoms (within 5 days of onset). A total of 23 volunteers who met the inclusion criteria were enrolled to reach an initial target of 15 confirmed COVID-19 positive adult subjects. All subjects received an initial mid-turbinate nasal swab to act as baseline for virus quantification prior to Nasodine treatment. This was followed by treatment with a single dose of Nasodine, comprising four sprays per nostril (1.12 mL total dose). Mid-turbinate swabs were then collected at 5, 15 and 60 minutes post-treatment to assess the impact on the viral titers compared with baseline (pre-treatment).

Because the *in vitro* testing had revealed that PVP-I can eliminate viral infectivity without affecting viral mRNA detected by PCR, it was essential to be able to culture the virus from the nasal swabs to assess the impact of PVP-I on viral infectivity *in vivo*. For this purpose, the swab samples were shipped on dry ice to PathWest Laboratory Medicine WA (PathWest) in Perth, Australia, where all cell culture and subsequent testing was performed. At PathWest, COVID-positivity was first confirmed using an in-house real-time RT-PCR test for three established SARS-CoV-2 RNA gene targets: Env-gene, S-gene 1 and S gene 2: Nucleic acid was extracted using the MagMAX magnetic bead kit on a semi-automated nucleic acid extraction instrument (MagMAX Express-96; Applied Biosystems) as modified by Sikazwe *et al.* (17). RT-PCR was performed in accordance with the method of Speake *et al.* (18). This confirmed that 14 of the 15 subjects diagnosed as COVID-positive in South Africa were positive via the PathWest method (Ct ≤40 one of the 3 gene targets). The ITT for further analysis was subsequently reduced to the 14 confirmed COVID-positive subjects.

Pooled swab samples from each nostril were cultured in triplicate from undiluted to 1/729 dilution in Vero E6 cell line. A laboratory grown confirmed B1.351 isolate at 100 $TCID_{50}$/mL was used as a positive control. CPE was scored visually following fixing and staining plates with 2% crystal violet in 10% formaldehyde and confirmed by testing of supernatant at day 10 via E-gene RT-PCR. Viral titers ($TCID_{50}$/mL) were calculated using the Reed Muench formula. The difference in titers of viable virus between baseline and swabs taken post-treatment was used as the treatment effect.

Statistical analysis

The primary endpoint was the reduction in the detectable amount of SARS-CoV-2 in PCR positive subjects as determined by viral titres (in $TCID_{50}$ units) associated with serial dilutions of the swab sample cultured in Vero cells for 96 hours. The standard deviation for the change in Ct values between cultured and uncultured sample is 4 (as informed by *in vitro* studies), and assuming a clinically significant minimal change of 4 Ct units, then a sample of at least 13 COVID PCR+ subjects will have >90% power to detect a significant decrease in the primary endpoint, using the paired t-test at the 5% level of significance. The frequency of positive COVID tests in the participants was estimated to be between 10–20% based on diagnostic testing data reported for South Africa (as at Sept 6 2020). To make provision for a 10% loss to follow-up (due to non-compliance, failure to complete the study, etc.), 15 COVID+ participants were required. Assuming 15% of the enrolled participants are found to be COVID+, the target enrolment was set at 100 (nQuery version 8.6 power and sample size calculator, Statsol, was employed)

Results

In vitro studies

In the first study, BetaCoV/Australia/VIC01/2020 was exposed to Nasodine (or control media) and either assayed immediately to determine a direct effect upon RNA copies or cultured for 96 hours in Vero cells to determine the effect upon virus viability. Based on Cycle threshold (Ct) scores measured immediately after the 60-second Nasodine treatment (0 h inoculum, *Figure 1A*), the number of RNA copies detected in the media control inocula (Alone) and the duplicate Nasodine-treated inocula were essentially identical across a 7-point dilution series (*Figure 1A*). This indicated that PVP-I exposure for 60 seconds did not affect (reduce) the detection of SARS-CoV-2 RNA by RT-PCR. In contrast, after growth for 96 hours in Vero cells following treatment (96 h, *Figure 1B*), the control inoculum displayed robust replication of SARS-CoV-2, as indicated by the lower Ct scores (higher viral RNA copies) compared to the uncultured 0 hour inoculum throughout the dilution series. The duplicate Nasodine plates showed no change in detectable RNA compared with the 0 hour inoculum and a linear relationship between Ct-values and dilution factor, indicating an absence of viral replication in tissue culture over 96 hours. In other words, the Nasodine treatment completely eliminated the infectivity of SARS-CoV-2, while not affecting PCR-detectable viral RNA.

In the second *in vitro* study, and compared with a saline control, Nasodine treatment resulted in a 3.5 $\log_{10}$ reduction in virus titer in 15 seconds, a 4.0 log reduction following a 5-minute exposure and no detectable viable virus (>4.3 log reduction) after 15-minute incubation (*Table 1*). The 15-second and 5-minute results were considered most relevant to clinical use because of likely rapid nasal clearance of the Nasodine, due to mucous secretions and mucociliary clearance which is estimated to be up to 20 minutes in normal circumstances.

In vivo study

Nasodine treatment was well tolerated, and all subjects completed the study. The baseline Ct values and overall culture outcomes for the 14 confirmed COVID-positive subjects (ITT population) are shown in *Table 2*.

Only 6 of the 14 PCR-positive samples yielded culturable virus (the efficacy subset). Of the 8 culture-negative samples, 7 had Ct scores above 30, which corresponded with extremely low levels of viral RNA present, i.e., 30 doubling cycles were needed to reach the positivity threshold. Given the well documented difficulty of culturing virus at Ct values above 25, it is not surprising that culturable virus could not be isolated from these samples.

Even in the 6 cases where virus could be cultured, the Ct scores were still high and indicative of low baseline virus levels, which were confirmed by the observed viral titers, making demonstration of any material antiviral effect challenging.

Five minutes after the administration of Nasodine, the mean viral titer was reduced by 65% versus baseline, and this was reduced further (79% versus baseline) at 60 minutes

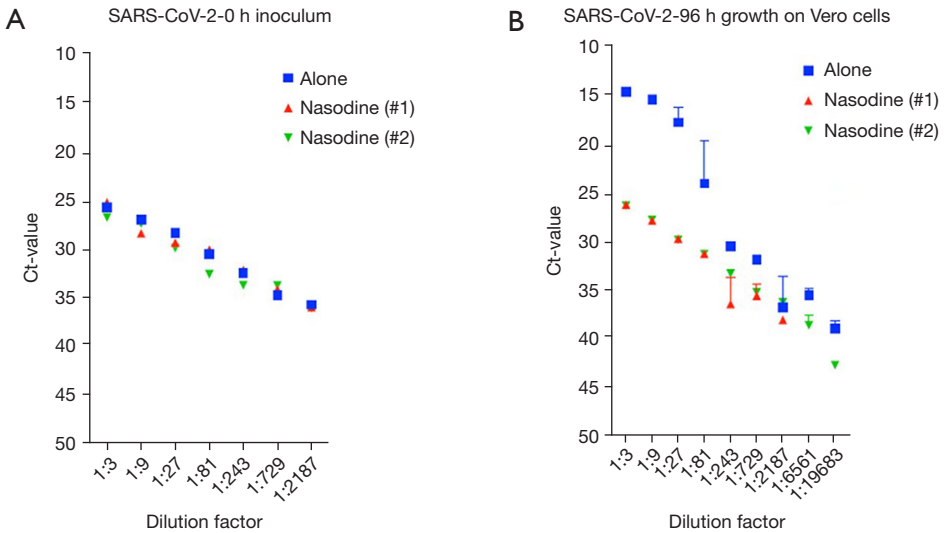

Figure 1 Titre of Nasodine (duplicates #1 and #2) and control treated SARS-CoV-2 via $TCID_{50}$ assay and RNA detection via real-time TaqMan RT-PCR. SARS-CoV-2 was exposed to the indicated test solution(s) for 1 minute before serial dilution (1:3) and incubation on Vero cells for either 0 hours or 96 hours. Values expressed as mean cycle threshold (Ct) value + SEM (n=3) versus dilution factor. (A) Time point zero (0 h) inoculum titration used to determine baseline Ct-values of treated samples prior to replication in Vero cells; (B) titres associated with cultures harvested 96 h post inoculation of Vero cells.

Table 1 Virucidal efficacy against SARS-CoV-2 after incubation with virus at 37 °C

Exposure time (min)	Virus titer[a]		
	Nasodine	Saline	Nasodine LRV[b]
0.25	1.5	5.0	3.5
5	1.0	4.5	4.0
15	<0.67 (LOD)[c]	5.5	>4.3

[a], log_{10} $CCID_{50}$ of virus per mL, mean of 3 replicates; [b], LRV is the reduction of virus compared to the virus control; [c], LOD is the lower limit of this assay for detecting infectious virus. LRV, log reduction value; LOD, limit of detection.

post-dose. At 5 minutes post-dose, 5 of the 6 subjects (83%) were found to have either a reduction or cessation of viral shedding and at 60 minutes post-dose, virus titers were below the respective baseline values in all 6 subjects.

The data are summarized in *Table 3* and *Figure 2*.

All 6 cases, regardless of the culture result, the samples remained PCR-positive for SARS-CoV-2. In all 6 cases, the culturable virus was shown by whole genome sequencing to be the VOC beta/B.1.351 variant, first described in South Africa.

Discussion

The *in vitro* activity of PVP-I against SARS-CoV-2 has been established for some time and the *in vitro* studies reported here confirm the activity of Nasodine Nasal Spray (0.5% PVP-I), a commercial PVP-I nasal spray, in timeframes that are relevant to nasal utility. What is most surprising about the *in vitro and in vivo* results is not the potent, rapid activity of the microbicide on the enveloped virus, but the lack of effect on PCR-detectable viral RNA, these observations are consistent with the clinical findings of Lamas and colleagues who reported that PVP-I based mouthwash reduced SARS-CoV-2 RNA copies in COVID-19 patients' saliva at 1–3 hours post treatment but not at the earlier time point of 5 minutes post treatment (19). We hypothesize that this difference is a consequence of clearance of RNA from PVP-I inactivated virus between these time points by endogenous mechanisms such as mucociliary clearance (estimated to be up to 20 minutes

Table 2 Ct values and culture results at baseline for COVID-positive subjects

Subject No.	Ct Env gene	Ct S gene 1	Ct S gene 2	Culture
1	34.68	33.90	32.8	–
3	24.7	25.9	26.7	+
5	23.5	25.2	25.9	+
6	–	–	–	–
7	32.49	34.41	33.21	–
8	31.0	31.9	33.3	+
10	30.0	31.2	29.1	+
16	30.2	31.4	32.0	–
17	37.1	34.9	38.2	–
18	25.6	26.8	28.1	+
20	37.9	–	–	–
21	24.2	26.3	28.1	+
22	–	43.0	–	–
23	36.86	35.06	36.90	–

Table 3 Viral titers for culturable samples

Time after Nasodine treatment	Subject No ($TCID_{50}$ per mL)					
	3	5	8	10	18	21
Baseline	45.6	137	11.5	8.8	34.6	104
5 min	11.5	45.6	–	–	6.7	137
15 min	–	104	–	–	–	137
60 min	–	104	–	–	6.7	34.6

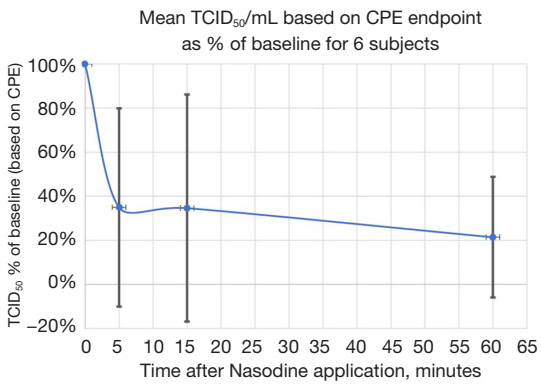

Figure 2 Mean $TCID_{50}$/mL as % of baseline for 6 subjects, based on CPE endpoint.

under normal circumstances but may be longer in infected individuals).

This is an important finding in the context of several studies currently underway to assess the effectiveness of various PVP-I nasal formulations in the COVID-19 setting. One of the implications of this is that for studies of the effect of PVP-I on shedding of SARS-CoV-2, PCR alone cannot be relied upon for measuring the effect of PVP-I nasal or oral formulations on virus infectivity. Culture is needed in all cases.

Another implication is that while intranasal 0.5% PVP-I may temporarily reduce or interrupt nasal shedding of SARS-CoV-2, it does not affect the detection of SARS-

CoV-2 RNA using PCR based methods (nor did it cause false positive results). This is also an important finding, because if intranasal PVP-I were utilized in either infected or non-infected symptomatic or asymptomatic patients, it would not be expected to compromise PCR-based COVID-19 testing. Although the factors governing nasal deposition patterns are multi-faceted and the delivery to the site at which most preferred diagnostic nasopharyngeal swab samples are taken is not yet known, we hypothesize that deposition of PVP-I in the nasopharynx may also be lower than the anterior or mid-turbinate regions.

In the *in vivo* study, the fact that 8 out of 14 confirmed COVID-positive samples did not yield viable virus for cell culture may reflect just how sensitive the PCR test is, in that it will detect minute quantities of viral RNA, or RNA fragments, and does not necessarily reflect the presence of viable virus. Alternatively, this could also reflect certain study design limitations discussed below.

All culturable virus from the human *in vivo* study was confirmed by whole genome sequencing to be the VOC beta/B.1.351 variant, first described in South Africa. These results represent the first report of the confirmed activity of PVP-I *in vivo* against this variant that has recently been described to harbor mutations that may mediate vaccine escape from vaccine responses (20,21).

Overall, the results provide evidence of rapid inactivation of SARS-CoV-2 at a clinically tolerable concentration and a signal of a favorable effect of 0.5% PVP-I nasal spray on nasal shedding of SARS-CoV-2 in humans. These findings warrant larger clinical studies.

Study limitations

There were a number of limitations in the *in vivo* study. It was a small single arm pilot study designed to obtain signals of activity *in vivo* and elucidate considerations for further clinical studies. These learnings included logistical challenges, including recruitment of COVID-19 patients sufficiently early in the disease and symptomatology, such that nasal shedding was sufficiently high to yield culturable virus. The study was not intended to and did not provide any indication of how long the effect of Nasodine might be sustained after 60 minutes or the effect of multiple doses. The subjects were all found to be infected with VOC beta/B.1.351 variant, first described in South Africa. We cannot know how well these results may translate to other variants, but PVP-I is known to be universally active against many different virus species so there is no expectation that the inactivation results will not be relevant for other variants. We also note that the *in vitro* data were generated with other variants

Further, during the pandemic, it was extremely difficult to find any laboratories that were not inundated with samples for routine testing and had the capacity and required biosafety level to partake in research analysis for viral culture. This resulted in an additional layer of logistical challenges and delays related to separate and individual NHMRC, Western Australian and PathWest Human Research Ethics approvals together with various Australian Government Department of Agriculture and Fisheries licenses and approvals for importation of samples.

Conclusions

The *in vitro* and *in vivo* data provide encouragement for further evaluation of PVP-I nasal spray as an adjunct in the management of COVID-19. Larger scale confirmatory trials are warranted, to determine whether repeated usage over 5 or 10 days leads to sustained suppression of viral shedding and transmission, and whether it has an impact on clinically meaningful disease outcomes.

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Footnote

Reporting Checklist: The authors have completed the TREND reporting checklist. Available at https://ajo.amegroups.com/article/view/10.21037/ajo-21-40/rc

Data Sharing Statement: Available at https://ajo.amegroups.com/article/view/10.21037/ajo-21-40/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure (available at https://ajo.amegroups.com/article/view/10.21037/ajo-21-40/coif). Dr. PF, ST, SG and PM are all affiliated with Firebrick Pharma Ltd. Dr. PF serves as an unpaid editorial board member of *Australian Journal of Otolaryngology* from January 2021 to December 2022. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are 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. The trial was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Sir Charles Gairdner and Osborne Park Health Care Group Human Research Ethics Committee (EC 00271/RGS0000004334) and South African Pharma -Ethics (200923548) and South African Health Products Regulatory Authority (20200439) and informed consent was taken from all individual participants.

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