Effects of Intra-Nasal Calcium Glycerophosphate on Olfactory Recovery in Post-Viral Smell Loss on Mouse-Adapted H1N1 Flu Virus Strain – Influenza A/Puerto Rico/8/34 (PR8) Infected Mice

FINAL REPORT

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Summary:

Respiratory viral infections are leading causes of olfactory loss. The COVID-19 pandemic has highlighted the prevalence of viral-induced smell loss: a large number of COVID-19 patients have experienced smell loss and 5-20% of them may develop long-term loss. Besides COVID-19, other respiratory viral infections, such as flu, also lead to long-term smell loss in some patients. Olfactory loss, especially long-term loss, is a serious health issue, not only affecting quality of life but also leading to anxiety, depression and malnutrition. Currently, there is no effective and specific treatment for viral-induced smell loss.

In this study we tested whether calcium glycerophosphate (CGP) could promote olfactory recovery in post-viral smell loss. CGP is an alkaline phosphatase inhibitor that is conjectured to be able to attenuate dephosphorylation of sphingosine-1-phosphate (S1P) and thus upregulate the level and longevity of endogenous S1P, a multifunctional molecule that promotes neural survival, differentiation, and regeneration. Although CGP has been previously demonstrated these properties in various studies, its effects on the olfactory sensory system are unknown.

Here we used a preclinical viral-induced smell loss model in mice to test the effects of CGP on olfactory recovery. Mice were infected intranasally with a mouse-adapted H1N1 influenza virus strain. At 30 days post infection, all infected mice showed significantly impaired olfactory function in behavioral tests. One group of mice was then treated with 3.75% CGP intranasally once every two days for a total of 8 doses. Control mice were given a placebo (sterile water) intranasally. Mice were then tested for olfactory performance in behavioral tests. Olfactory tissues were collected and analyzed for morphological changes, if any, for possible correlation of same with behavioral changes, if any.

Our results showed that, in buried food finding tests, it took about the same length of time for CGP-treated mice and placebo control mice to find the buried foods, a task that mostly relied on olfactory function. Post-treatment histological observations showed that the gross structure of the olfactory epithelium remained similar in control and CGP-treated mice, while in CGP-treated mice only, some regions of the olfactory epithelium did exhibit morphological alterations that were consistent with mild tissue disturbances. The exact nature of these morphological changes in CGP-treated mice remains to be determined. Overall, our data did not show significant improvement in olfactory function by CGP treatments in mice with post-viral smell loss.

Experimental procedures:

1. Viral-induced smell loss model.

(1). Respiratory virus.

The mouse-adapted H1N1 flu virus strain – influenza A/Puerto Rico/8/34 (PR8) was used in this study. All experiments using the virus were conducted in a biosafety level 2 (BSL2) facility. The study was approved by the Monell Institutional Biosafety Committee. The approved biosafety guidelines were strictly followed.

(2). Mice.

All experiments involving mice were approved by the Monell Institutional Animal Care and Use Committee. We used a knockout (KO) mouse strain that lacked the functional IRF3 gene. IRF3 is a key factor in several anti-viral pathways and is involved in the production of type I interferons, critical signaling proteins that orchestrate anti-viral immune responses. The IRF3 KO mouse strain in our lab was originally generated in the laboratory of Dr. T. Taniguchi [Sato et al. 2000. Immunity 13(4):539-548]. It was a conventional knockout strain that resulted in the deletion of the putative transcriptional initiation site and the coding exon of the N-terminal part of the IRF3 DNA binding domain. These mice have been backcrossed to C57BL/6J mice for several generations.

Four groups of mice were used: **Group 1**, noninfected KO mice receiving placebo (sterile water); **Group 2**, noninfected KO mice receiving CGP; **Group 3,** infected KO mice receiving placebo (sterile water); and **Group 4**, infected KO mice receiving CGP.

(3). Infection.

Mice were anaesthetized by intraperitoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine (5mg/kg). When mice were deeply anaesthetized, 8 µl of a viral suspension in phosphate-buffered saline (PBS) was administered intranasally into each nostril of anesthetized mice.

Noninfected mice were given 8 µl of PBS per nostril.

2. CGP and placebo administration.

(1). CGP formulation.

CGP was provided by AkPharma Inc. A 3.75% suspension in sterile water was used.

(2). Placebo.

Sterile water was used as the placebo.

(3). Administration.

CGP and placebo were administered intranasally. Mice were anaesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5mg/kg) before administration of

CGP or placebo. Administration was done once every two days for a total of 8 doses. Each administration was 10 µl of liquid test product CGP solution per nostril.

3. Olfactory analysis.

(1). Food-finding olfactory performance test.

The evening before food finding tests, mice were placed on a restrictive diet of about 0.3 gram of a piece of cheese cracker. Water was given ad libitum.

Each test session included 4 trials per mouse. The first trial was an open-food test, i.e., a piece of cheese cracker was placed on the surface of the bedding. The following 3 trials were buried-food tests, i.e., a piece of cheese cracker was buried under the bedding.

The tests were carried out in standard mouse cages. The spaces in the mouse cages were assigned into 4 quadrants that were divided at the center of the cage. A piece of cracker was placed on a clean piece of paper towel in a random quadrant of a clean mouse cage. For buried-food tests, a piece of cracker was buried under about ½ inch clean shavings. Care was taken to avoid evidence-delivering "bumps" in cage floors above the food caches that would deliver signals other than olfactory. To ensure no bias from the investigators, each quadrant was pre-determined using RANDBETWEEN function in Excel to generate random numbers (1-4) before testing.

To start each trial, the mouse was placed in the center of the cage and quietly observed for active searching (walking around the cage, sniffing and digging). Time started once the mouse was in the cage and ended when the mouse found the cracker. The mouse was allowed to finish the found cracker and then placed back to its home cage. The bedding over and surrounding the piece of cracker and the piece of paper towel were then removed and discarded. A new clean cage was used for each mouse.

Mice in all 4 groups were tested with this food finding tests at 30 days after viral infection to monitor their smell loss. After confirming smell loss, mice were given either placebo or CGP for a total of 8 doses. Then each mouse was tested again in 2 sessions (1 session within the first week after CGP or placebo administration, and another session within the second week of CGP or placebo administration).

Data from the 4 groups of mice were analyzed and compared.

(2). Histological analysis.

After the food finding experiment, mice were euthanized for histological analysis of the olfactory tissues. Mice were perfused with PBS and 4% paraformaldehyde (PFA) in PBS. Olfactory tissues were removed and post-fixed in 4% PFA/PBS for overnight. The tissues were then decalcified using an EDTA/HCl decalcification solution for 5-7 days. The tissues were then cryoprotected in 20-30% sucrose and mounted in tissue mounting medium. Frozen tissues were sliced into thin sections using cryostat equipment. The morphology of olfactory tissues was examined by H&E staining.

Data from the 4 groups of mice were then analyzed and compared.

Results:

1. Mice showed impaired olfactory performance after influenza virus infection in food finding tests.

Before CGP or placebo administration, we evaluated whether influenza virus infection caused significant smell loss in infected mice at 30 days post infection (30 dpi). Buried-food finding tests showed that mice infected with the virus took significantly longer time to find the food than did the noninfected mice (Figure 1), suggesting impaired olfactory performance.

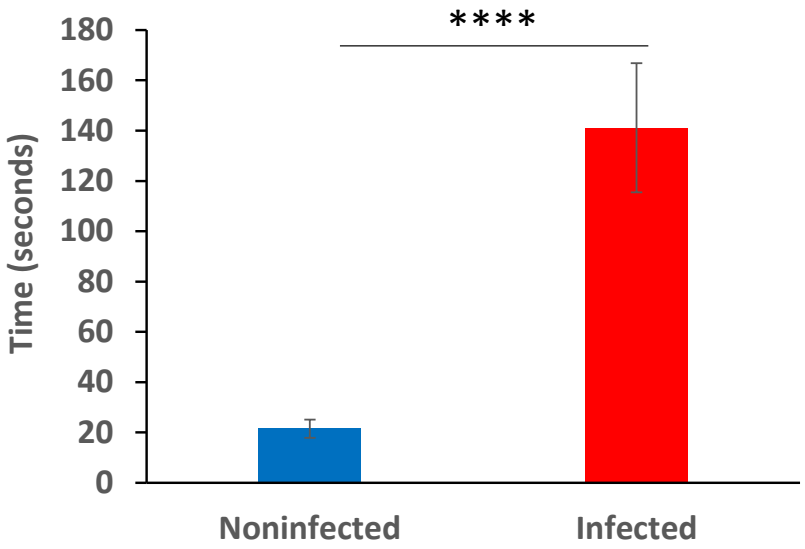

Figure 1. Buried-food finding tests at 30 dpi showed impaired olfactory performance of flu virus-infected mice. The tests were performed before CGP or placebo administration. Noninfected: including mice from Groups 1 and 2, N=19. Infected: including mice from Groups 3 and 4, N=10. ****: $p < 0.00001$.

2. Mice treated with CGP did not show improved olfactory performance compared to placebo control mice in food finding tests.

After 8 doses of CGP or placebo administration, we performed 2 sessions of food finding tests (within the first and second week after the administration, respectively) to evaluate whether CGP could improve olfactory performance. As shown in Figures 2A and 3A, at these time points, infected mice still showed significantly impaired performance compared with noninfected mice. Further, compared to placebo controls, CGP-treated mice did not show any improved performance in these tests (Figures 2B and 3B).

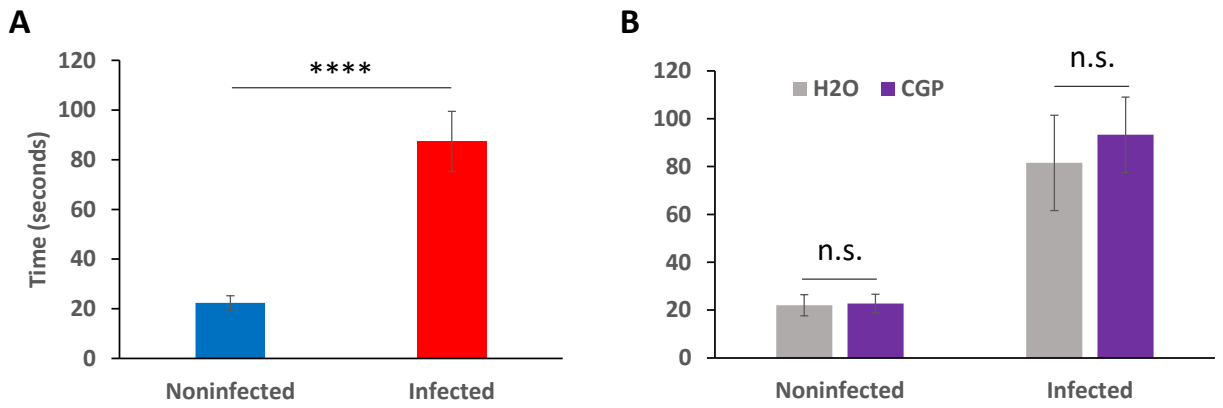

Figure 2. Buried food-finding tests within the first week post CGP administration. (**A**) Infected mice (Groups 3 and 4, N=10) took significantly longer time to find buried food than noninfected mice (Groups 1 and 2, N=19). ****: $p<0.00001$. (**B**) CGP-treated mice did not show improved performance in buried food-finding tests compared to placebo controls. Group 1 (noninfected, H2O): N=10; Group 2 (noninfected, CGP): N=9; Group 3 (infected, H2O): N=5; and Group 4 (infected, CGP): N=5. n.s.: not significant.

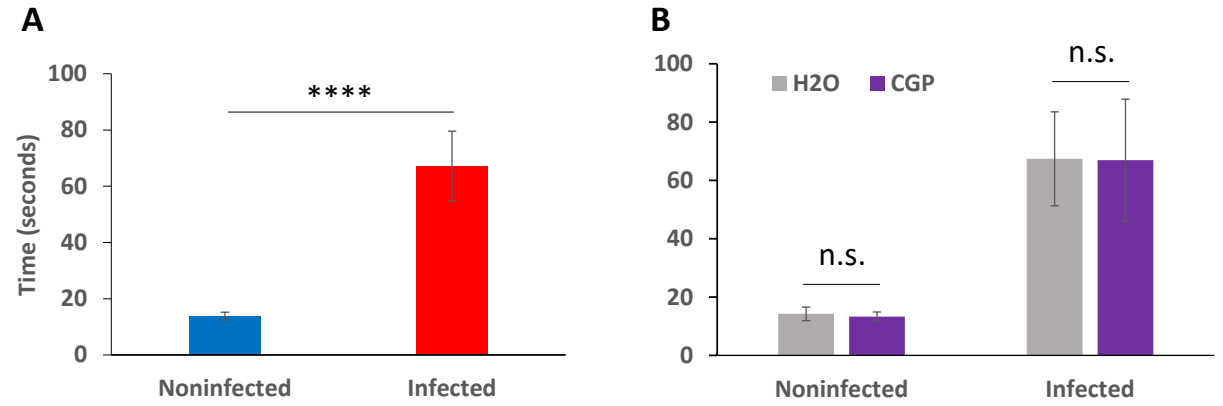

Figure 3. Buried food-finding tests within the second week post CGP administration. (**A**) Infected mice (Groups 3 and 4, N=10) still took significantly longer time to find buried food than noninfected mice (Groups 1 and 2, N=19). ****: $p<0.00001$. (**B**) CGP-treated mice did not show improved performance in buried food-finding tests compared to placebo controls. Group 1 (noninfected, H2O): N=10; Group 2 (noninfected, CGP): N=9; Group 3 (infected, H2O): N=5; and Group 4 (infected, CGP): N=5. n.s.: not significant.

3. The gross structures of the olfactory epithelium (OE) were similar in CGP-treated mice and placebo control mice, although some regions of the OE in CGP-treated mice showed morphological changes.

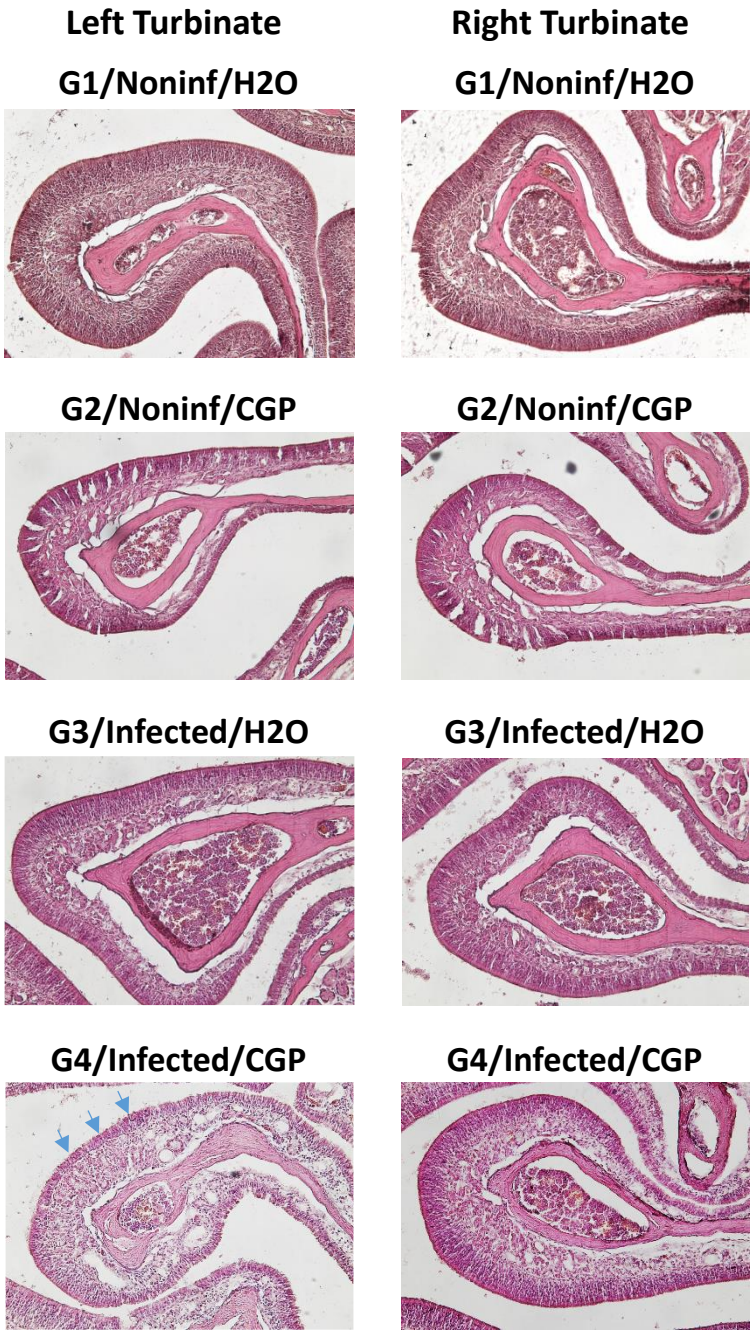

Figure 4. H&E staining of the olfactory epithelium (OE). Representative images of the left and right turbinate from the anterior part of the OE are shown. Some regions of the OE from Group 4 mice (infected, CGP-treated) showed morphological changes in the epithelium (blue arrows).

H&E staining showed that the gross structures of the OE were similar in CGP-treated mice and placebo control mice (Figure 4). However, some regions of the OE in CGP-treated mice exhibited morphological changes that suggest mild structural disturbances (Figure 4, blue arrows). The exact nature and implications of these structural changes are unclear.

Conclusions:

In this study we investigated whether CGP could promote olfactory recovery after viral-induced smell loss. We used an influenza virus-induced smell loss model in mice. Our results showed that influenza virus infection resulted in a significant reduction in olfactory performance of infected mice compared to noninfected mice. Post-infection CGP administration did not improve olfactory performance of CGP-treated mice compared to placebo controls. The gross structures of the OE were similar in CGP-treated mice and placebo control mice, although some morphological changes were noted in the OE of CGP-treated mice. The exact nature and implications of these structural changes remain to be determined and suggest future investigation.
