PHYSIOLOGICAL FACTORS MODULATING BACTERIAL SECOND MESSENGERS AND THE COMPARISON OF ANTIMICROBIAL EFFICACY OF ENDODONTIC IRRIGANTS

PhD thesis

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INTRODUCTION

Microorganisms respond to environmental cues and hostile conditions by changing their modes of life between biofilm formation and planktonic lifestyle. Biofilm is the preferred lifestyle in most bacterial species found in the environment. The transition between biofilm and planktonic lifestyle of bacteria is orchestrated by alterations of intracellular second messengers levels including c-di-GMP, cAMP and c-di-AMP, etc. Increased c-di-GMP and decreased cAMP concentrations involve in biofilm formation as they reduce the motility of bacterial cells and enhance the production of extracellular polymeric substances (EPS). The recent c-di-AMP is also discovered to play an important role in biofilm formation, particularly Gram-positive bacteria.

Bacteria have the ability to attach to a wide range of surfaces forming biofilms, such as living tissue, implantable medical devices, and industrial water system piping. Therefore, biofilms are considered to be a principal problem at present, especially in medical field, because they are strongly resistant to various antibiotics resulting in poor clinical outcomes in patients with chronic infections.

Biofilm formations by *Pseudomonas aeruginosa* and other pathogens in cystic fibrosis (CF) lead to increased morbidity and mortality of the patients. Regardless of the virulence of biofilm itself, the attenuated antibacterial functions, impaired immune response and thick mucus blockage in airway surface liquid (ASL) provide favourable environment for biofilm formation. Bicarbonate does not only maintain the equilibrium in ASL but may also play an important role in antimicrobial activity in the

airway. Thereby, we aimed to test antibacterial effect of HCO_3^- on second messenger levels of *P. aeruginosa* related to biofilm formation.

In addition to cystic fibrosis, we extend our investigation to second messengers of oral bacteria, *Streptococcus mutans*, because the biofilm formation by aciduric bacteria is one of the major causes of dental caries. *S. mutans* biofilm can produce a large amount of EPS, utilize various carbohydrates into acid and survive under extremely low pH. However, there is a lack of knowledge of how aciduric bacteria respond to different pH conditions by modulating their second messenger. Consequently, we tested the change of second messengers including recent c-di-AMP of *S. mutans* under different pH values exposure.

Despite dental caries, root canal infections and failure of endodontic treatment are mainly caused by persistent biofilm formation in the root canal. Consequently, the primary goal of endodontic treatment aims to remove bacteria and their by-products from the root canal system and periapical tissues. To achieve this goal, efficient mechanical preparation combined with root canal irrigation is recommended for root canal disinfection. However, the disagreement in antimicrobial efficacy among the most frequently used root canal irrigants, sodium hypochlorite (NaOCl) and chlorhexidine (CHX), still exists. Our objective is to compare the antimicrobial effect of these two irrigants by performing a systematic review of randomized clinical trials (RCTs) supplemented with a meta-analysis.

OBJECTIVE

1 We aimed to investigate the effects of HCO_3^- on second messenger concentrations and biofilm formation of *Pseudomonas aeruginosa*.

Our study aimed to explain the antimicrobial effect of HCO_3^- on the most prevalent bacteria in cystic fibrosis airway, especially *P. aeruginosa*. Furthermore, we aimed to develop a protocol to simultaneously investigate c-di-GMP and cAMP levels related to their biofilm formation and to investigate the biofilm formation during HCO_3^- supplementation in real-time.

2 Our aim was to investigate the effects of different pH values on second messenger concentrations and biofilm formation of *Streptococcus mutans*.

We aimed to study how *S. mutans* responds to extracellular alkaline and acidic pH by changing its second messengers, including the c-di-GMP, cAMP and recently discovered c-di-AMP, related to the growth and biofilm formation.

3 We aimed to compare the clinical antimicrobial efficacy of the most frequently used root canal irrigants, NaOCl and CHX.

Our systematic review supplemented with meta-analysis aimed to study the antimicrobial efficacy of NaOCl and CHX during root canal therapy, as well as other related factors such as type of irrigants, concentration and exposure time from the available RTCs.

METHODS

1. Antimicrobial effect of bicarbonate on *P. aeruginosa*

1.1 Determination of intracellular cAMP levels based on an ELISA-based method

We first investigated the effect of HCO_3^- on cAMP levels in *P. aeruginosa* and *Staphylococcus aureus*. The bacteria were incubated in a brain-heart infusion (BHI) medium supplemented with $NaHCO_3$ or NaCl for 16 h. The pH of BHI medium alone and BHI media with NaCl were set to 7.4 by HCl or NaOH, whereas the $NaHCO_3$ groups were equilibrated with CO_2 incubation to maintain the desired pH. The cAMP levels were subsequently measured by Cyclic AMP XP® Assay Kit. The bacteria were grown in the desired medium for 15–16 h, then diluted in the same fresh media and allowed to them grow for a few hours until reaching log phase. Finally, the supernatant was transferred to the assay plate and subsequently measured at 450 nm.

1.2 Determination of intracellular c-di-GMP and cAMP Levels based on an HPLC-MS method

We further investigated the effect of HCO_3^- on c-di-GMP parallel with cAMP as following new conditions; 1) BHI alone pH 7.4; 2) BHI + 25 mM NaCl pH 7.4; 3) BHI + 25 mM NaHCO3 pH 7.4; 4) BHI + 50 mM NaCl, pH 7.7; and 5) BHI + 50 mM NaHCO3 pH 7.7. The cultures in BHI media with NaCl were incubated at 37 °C in ambient air, while cultures in added $NaHCO_3$ media were incubated in the presence of 5% CO_2.

The bacteria were grown in the designated media in the condition described above. After 16 h incubation, the extraction was performed as it was modified from Petrova and Sauer's

protocol in 2017. Second messengers in the samples were subsequently determined by the HPLC-MS method. The remaining cell pellets were used for protein measurement by using a Quant-iT™ protein assay. Finally, the total protein content was used to normalize the c-di-GMP and cAMP levels obtained from the HPLC-MS method. Three independent bacterial cultures were performed in all cases.

1.3 Measurement of biofilm formation assessed by cell-impedance based method

To measure the biofilm formation, we inoculated the diluted *P. aeruginosa* in 96-well E-plates equipped with a xCELLigence Real-time cell analyzer (RTCA) in 5% CO_2 incubator. The RTCA can detect the attachment of bacterial cells. Bacteria were inoculated in different designated media including BHI medium alone, BHI supplemented with either 25 or 50 mM NaCl, and 25 or 50 mM $NaHCO_3$. The pH of media supplemented with NaCl (25 or 50 mM) was adjusted with NaOH to pH 8.0 or pH 8.4; however, these pH values reduced to desired pH 7.4 and 7.7, respectively, after 5% CO_2 incubation for 48 h. After the inoculation, cell impedance signals were recorded every 10 min for 48 h. The signals obtained at 6, 12, 24 and 48 h were converted by the xCELLigence software to delta cell indices (ΔCI). The measurements were parallelly performed 4-5 times.

1.4 Measurement of biofilm formation assessed by a crystal violet assay

We grew *P. aeruginosa* in 96-well microtiter plates. BHI media were prepared as described in the cell impedance-based method. The plate was incubated for 48 h at 37 ◦C in 5% CO_2. The media

and planktonic cells were carefully discarded and then washed by PBS. We added crystal violet to each well for staining the adherent cells and biofilms. Finally, acetic acid was added to each well to dissolve crystal violet and transferred to a new plate for absorbance measurement. Three independent biological cultures were performed.

2. Effect of pH on second messengers and biofilm formation in *S. mutans*

2.1 Effect of different pH values on second messengers in *S. mutans*

To test the effect of different pH values on the second messenger in *S. mutans*, we grow *S. mutans* ATCC in BHI media at pH to 4.5, 5.5, 7.5 and 8.0. Bacteria were grown until they reached the stationary phase for 16 h and then transferred to expose designated media with different pH values for 0.5 or 3 h. The extraction was immediately performed as the same protocol as in *P. aeruginosa*. We measured c-di-AMP in addition to c-di-GMP and cAMP.

2.2 Effect of different pH values on biofilm formation of *S. mutans*

S. mutans ATCC were inoculated in 96 wells-plate with BHI media at pH 4.5, 5.5, 7.5 and 8.0. The biofilm formation was assessed by crystal violet assay after 48 h incubation as described protocol above.

2.3 Statistical analysis

Normalized c-di-GMP, cAMP and c-di-AMP concentrations were calculated by Microsoft Excel 2019 using a previously described formula. All data are presented as mean ± SD. One-way ANOVA

or TWO-way ANOVA was used for statistical analysis followed by a Tukey's multiple comparison test. GraphPad Prism version 8.0.0 was used for calculation and analysis. Significance was accepted at $p < 0.05$.

3. Comparison of the effectiveness of NaOCl and CHX in root canal irrigation

3.1 Search and selection of studies

A systematic search was performed until March 2020 from 4 databases. Reference lists from the identified studies were also searched for additional studies in the English language. The search strategy complied with PICO components and selection criteria as follows:

P – Population: participants with pulpal and/or periapical disease receiving endodontic treatment in permanent teeth; Intervention, CHX irrigant

C – Comparison: NaOCl irrigant

O – Outcome: the primary outcome was reduced bacterial amount and incidence of positive bacterial samples after irrigation, whereas the secondary outcome was an improvement of clinical symptoms, radiographic parameters, and postoperative pain;

S – Study type: Randomized clinical trials (RCTs).

Our inclusion criteria were randomized controlled trial studies (RCTs) that applied irrigants in permanent teeth with pulpal and/or periapical disease during endodontic treatment. These studies compared the antimicrobial effects between CHX and NaOCl irrigants and reported the outcome as bacterial reduction by using bacterial culture and/or molecular microbiological methods.

3.2 Statistical analysis for meta-analysis

Relative risk (RR) was calculated for studies that reported samples showing positive and negative bacterial growth after irrigation. Standardized mean difference (SMD) was calculated for studies that reported the number of bacteria before and after irrigation. The 95% confidence intervals (CIs) were calculated for RR and SMD. In case of sufficient data, subgroup analysis was conducted. The significance of any variation and degree of heterogeneity was determined by I^2 and chi-square statistics, respectively. Pooled estimates were calculated with a random effects model using the DerSimonian-Laird method. We used a comprehensive Meta-Analysis Software Version 3 to compute the RR and SMD.

RESULT

1. Antimicrobial effect of bicarbonate on *P. aeruginosa*

1.1 Bicarbonate and alkaline pH increased intracellular cAMP levels based on an ELISA-based method

The BHI medium with 100 mM $NaHCO_3$ significantly increased cAMP production in *P. aeruginosa* and *S. aureus* compared to the absolute control (BHI media alone) and BHI media with the same osmolarity (100 mM NaCl) after 16 h incubation. In *S. aureus*, the effect of 25 and 100 mM $NaHCO_3$ was detected compared to the BHI media alone. Notably, the supplementation of 100 mM NaCl in BHI media did not affect cAMP concentrations in both *P. aeruginosa* and *S. aureus* compared to the absolute control.

In addition, various pH values ranging as 6.0, 6.8, 7.4 and 9.0 were tested on cAMP production. Alkaline pH values slightly

increased cAMP levels stepwise from lower pH to higher pH values. Interestingly, the cAMP concentrations induced by the highest pH value (9.0) were significantly lower than those caused by 100 mM $NaHCO_3$. Taken together, increased cAMP levels are mainly due to HCO_3^-*per se.*

1.2 Bicarbonate decreased intracellular c-di-GMP and increased cAMP Levels based on an HPLC-MS method

The control groups (NaCl) were compared to treatment groups ($NaHCO_3$) at the equivalent osmolarity and pH. In *P. aeruginosa* ATCC, both 25 and 50 mM $NaHCO_3$ significantly reduced c-di-GMP concentrations compared to the 25 and 50 mM NaCl at the same pH, respectively. In *P. aeruginosa* clinical isolate, only 50 mM $NaHCO_3$ reduced c-di-GMP levels compared to 50 mM NaCl at the same pH. These results suggested that osmolarity and pH had no considerable effect on c-di-GMP levels. The c-di-GMP concentration further decreased from 25 mM to 50 mM $NaHCO_3$ indicating a dose-dependent effect of HCO_3^- in both ATCC and clinical isolate.

Regarding cAMP levels in both *P. aeruginosa* ATCC and clinical isolate, 25 and 50 mM $NaHCO_3$ significantly increased cAMP levels compared to NaCl groups with equal concentrations and pH values. We also detected the does-dependent effect of HCO_3^- on cAMP levels in ATCC strain clinical isolate as the cAMP levels increased from BHI 25 mM to 50 mM $NaHCO_3$. In addition, administration of BHI medium with 25 or 50 mM NaCl or pH changes did not significantly affect the intracellular cAMP levels.

Taken together, HCO_3^- increases cAMP, while decreases c-di-GMP concentrations in dose-dependent manner. These changes are not caused by the alterations of osmolarity and pH.

1.3 Effects of bicarbonate on biofilm formation based on a cell-impedance based method

Delta cell indices (ΔCI) in clinical isolate vastly increased at 48 h suggesting significantly strong biofilm formation. The effect of HCO_3^- was investigated in BHI medium supplemented with either $NaHCO_3$ (25 or 50 mM) compared to control media (NaCl 25 or 50 mM). The ΔCI of clinical isolate in BHI medium alone and NaCl groups at 48 h dramatically increased compared to previous time points. However, both 25 and 50 mM $NaHCO_3$ significantly reduced ΔCI compared to the equal concentrations of NaCl following 48 h incubation.

1.4 Effects of sodium bicarbonate on biofilm formation assessed by a crystal violet assay

In *P. aeruginosa* ATCC, both 25 and 50 mM $NaHCO_3$ did not decrease biofilm formation as judged by the crystal violet assay. Nonetheless, 50 mM $NaHCO_3$ significantly reduced biofilm formation compared to NaCl group with the equal concentration and pH in *P. aeruginosa* clinical isolate. This comparison indicated that the alterations in osmolarity or pH did not play a crucial role in the inhibitory effect on biofilm formation by $NaHCO_3$.

2. Effect of pH on second messengers and biofilm formation in *S. mutans*

2.1 Effect of different pH values on second messengers in *S. mutans*

We extracted and measured c-di-GMP, cAMP and c-di-AMP levels after exposure to pH 4.5, 5.5, 7.5 and 8.0 for 0.5 and 3 h. All normalized second messengers showed no significant difference between 0.5 and 3 h exposure time, except the c-di-AMP levels at pH 8.0 which were significantly different between two-time points. These data indicate that incubation time has no influence on these second messenger levels under different pH exposure.

At both incubation periods, BHI pH 8.0 significantly reduced c-di-GMP concentrations compared to pH 7.5. On the contrary, acidic pH values did not alter c-di-GMP levels.

For the cAMP change, no significant difference was observed at 0.5 h due to various pH values exposure, but it decreased in a stepwise manner from pH 5.5 to the more alkaline pH values at 3 h incubation time. Although there was no significant difference of cAMP levels at 0.5 h, cAMP levels under pH 8.0 at 3 h were significantly lower than cAMP levels under acidic pH 4.5 and 5.5.

In addition, c-di-AMP levels similarly changed as c-di-GMP under pH alterations. However, overall concentrations of c-di-AMP are essentially lower than c-di-GMP. C-di-AMP levels decreased both in the acidic and in the basic directions compared to pH 7.5 following 0.5 and 3 h.

2.2 Effect of different pH values on biofilm formation of *S. mutans*

Biofilm formation of S. mutans ATCC was assessed by crystal violet assay after 48 h incubation. The data showed that alkaline pH 8.0 increased biofilm formation, while more acidic pH values (pH 4.5 and 5.5) decreased biofilm formation following 48 h compared to pH 7.5. However, there was no significant difference between pH 4.5 and 5.5.

2.3 Effect of different pH values on biofilm formation of *S. mutans* after 48 h

The result showed control pH 7.5 and alkaline pH 8.0 increased biofilm formation of *S. mutans* ATCC, while more acidic pH values (pH 4.5 and 5.5) decreased biofilm formation following 48 h. However, there was no significant difference between pH 4.5 and 5.5.

3. Antimicrobial efficacy of NaOCl and CHX irrigants

The pooled RR of samples with positive bacterial growth showed no significant difference in the incidence of positive samples in patients irrigated with CHX or NaOCl (RR=1.003, 95% CI: 0.729–1.380, p=0.987; heterogeneity: I^2=0.000%, p=0.673). This result suggested no significant differences between CHX and NaOCl treatments. The heterogeneities among studies were considered as low.

The SMD analysis indicated no significant difference (SMD=0.311, 95% CI: -0.368–0.991, p=0.3. However, these data were considerably heterogeneous (I^2=76.336%, p=0.005). Subgroup analysis did not show any significant difference between

2 methods (culture method: SMD=0.275, 95% CI: -0.765–1.315; molecular method: SMD=0.173, 95% CI: -0.636–0.982; p=0.880). The culture method revealed substantial heterogeneity (I^2=69.449%, p=0.070) whereas molecular method was considerable (I^2=81.463%, p=0.005).

CONCLUSION

1. **Investigating the effects of bicarbonate on second messengers and biofilm formation of *Pseudomonas aeruginosa***
   - In *P. aeruginosa*, which plays a crucial role in cystic fibrosis-related lung infections, HCO_3^- alone enhances cAMP and decreases c-di-GMP concentrations, thereby inducing a biofilm to planktonic state transition. These reciprocal changes in second messenger concentrations were not influenced by the medium pH or osmolarity. Our data demonstrate the antimicrobial properties of HCO_3^-, encouraging the use of aerosolized $NaHCO_3$ inhalation as a supportive treatment in CF patients.
2. **Investigating the effects of different pH values on second messengers and biofilm formation of *Streptococcus mutans***
   - In *S. mutans*, a notable cariogenic bacterium, environmental pH regulates its second messenger concentrations. Biofilm formations decreased with decreasing pH related to reduced c-di-AMP concentrations. On the other hand, alkaline pH reduced all second messengers, but increased biofilm formation. The environmental pH has an influence on all tested second messenger. However, further studies are required to find

explicit association between pH alterations and related second messenger and biofilm changes.

3. **Comparing the clinical antimicrobial efficacy of the most frequently used root canal irrigants, NaOCl and CHX**

- Our findings suggest that both NaOCl and CHX significantly, but not completely, reduce endodontic infections during root canal therapy. Based on our meta-analysis, no significant difference was found between the molecular and culture bacterial detection methods; therefore, both approaches are appropriate for detecting bacterial infections during root canal treatment.
- Both CHX and NaOCl were equally effective, despite their differing molecular mechanisms. Therefore, each of them can be used as the main antibacterial root canal irrigants. However, because a mixture of these two chemical substances can cause precipitate formation, we suggest a consecutive application with intermediate flushes between each irrigant and propose the development of more potent antibacterial agents.

The publications related to the PhD thesis (peer-review paper):

1) Ruksakiet K, Stercz B, Tóth G, Jaikumpun P, Gróf I, Tengölics R, Lohinai ZM, Horváth P, Deli MA, Steward MC, Dobay O, Zsembery Á. Bicarbonate evokes reciprocal changes in intracellular cyclic di-GMP and cyclic AMP Levels in *Pseudomonas aeruginosa*. Biology. 2021; 10(6):519. doi10.3390/biology10060519

SJR Scopus - Agricultural and Biological Sciences (miscellaneous): D1, IF: 5.079 (Expected IF value)

2) Dobay O, Laub K, Stercz B, Kéri A, Balázs B, Tóthpál A, Kardos S, Jaikumpun P, **Ruksakiet K**, Quinton PM, Zsembery Á. Bicarbonate inhibits bacterial growth and biofilm formation of prevalent cystic fibrosis pathogens. Front Microbiol. 2018 Sep 19;9:2245. doi: 10.3389/fmicb.2018.02245. PMID: 30283433; PMCID: PMC6157313.

SJR Scopus - Microbiology (medical): Q1, IF: 4.259

3) Ruksakiet K, Hanák L, Farkas N, Hegyi P, Sadaeng W, Czumbel LM, Sang-Ngoen T, Garami A, Mikó A, Varga G, Lohinai Z. Antimicrobial efficacy of chlorhexidine and sodium hypochlorite in root canal disinfection: A systematic review and meta-analysis of randomized controlled trials. J Endod. 2020 Aug;46(8):1032-1041.e7. doi: 10.1016/j.joen.2020.05.002. Epub 2020 May 12. PMID: 32413440.

SJR Scopus - Dentistry (miscellaneous): D1, IF: 4.171

The publications not related to PhD thesis (peer-review paper):

1) Jaikumpun P, **Ruksakiet K**, Stercz B, Pállinger É, Steward M, Lohinai Z, Dobay O, Zsembery Á. Antibacterial effects of bicarbonate in media modified to mimic cystic fibrosis sputum. Int J Mol Sci. 2020 Nov 16;21(22):8614. doi: 10.3390/ijms21228614. PMID: 33207565; PMCID: PMC7696793.

SJR Scopus - Computer Science Applications: D1, IF: 5.924

2) Keringer P, Farkas N, Gede N, Hegyi P, Rumbus Z, Lohinai Z, Solymar M, **Ruksakiet K**, Varga G, Garami A. Menthol can be safely applied to improve thermal perception during physical exercise: a meta-analysis of randomized controlled trials. Sci Rep. 2020 Aug 12;10(1):13636. doi: 10.1038/s41598-020-70499-9. PMID: 32788718; PMCID: PMC7423903.

SJR Scopus - Multidisciplinary: D1, IF: 4.380

The cumulative impact factor of the above published journals evaluated by Semmelweis University Central Library: 23.813 on 27.10. 2021, Budapest

Manuscript submitted for publication:

Levine M, Ruksakiet K, Földes A, Dinya E and Lohinai ZM. Genotypes detect strong and weak innate immunity phenotypes to oral bacteria in humans. Journal of Clinical Medicine by MDPI

Conference proceeding abstracts:

1) **Ruksakiet K**, Stercz B, Jaikumpun P, Dobay O, Zsembery Á, Tóth G, Horváth P, Lohinai Z. The effects of pH on second messengers in *S. mutans.* In: Proceeding of the CED-IADR/NOF Oral Health Research Congress. Brussels, Belgium (2021)
   Journal Article/Abstract (Journal Article)/Scientific
2) **Ruksakiet K**, Stercz B, Tóth G, Jaikumpun P, Dobay O, Horváth P, Zsembery Á, Lohinai Z. External pH regulates intracellular second messengers in *Streptococcus mutans.* In: Proceeding of the 68th ORCA Congress. Zagreb, Croatia (2021)
   Journal Article/Abstract (Journal Article)/Scientific
3) Levine M, **Ruksakiet K**, Földes A, Dinya E, Lohinai Z. Genes Control Gingival Crevicular fluid responsiveness to bacterial lysine decarboxylase. In: Proceeding of the 99th IADR General session, USA (2021)
   Journal Article/Abstract (Journal Article)/Scientific
4) **Ruksakiet K**, Stercz B, Tóth G, Jaikumpun P, Lohinai Z, Horváth P, Dobay O, Zsembery Á. Bicarbonate oppositely regulates cyclic di-GMP and cyclic AMP levels in *Pseudomonas aeruginosa.* In: Rakonczay, Zoltán; Kiss, Lóránd (eds.) Proceedings of the EFOP-3.6.2-16-2017-00006 (LIVE LONGER) project. Szeged, Hungary: University of Szeged (2020) 99 page 38
   Chapter in Book/Abstract (Chapter in Book)/Scientific
   Jaikumpun P, **Ruksakiet K**, Stercz B, Pállinger É, Steward MC, Lohinai Z, Dobay O, Zsembery Á. Sodium bicarbonate

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5) **Ruksakiet K**, Hanák L, Farkas N, Hegyi P, Sadaeng W, Czumbel L, Sang-ngoen T, Garami A, Mikó A, Varga G, Lohinai Z. Antimicrobial effectiveness of sodium hypochlorite and chlorhexidine irrigation: A meta-analysis Journal of dental research 98: B Paper: 0506 (2019)
Journal Article/Abstract (Journal Article)/Scientific
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Journal Article/Abstract (Journal Article)/Scientific
8) Zsembery Á, Jaikumpun P, **Ruksakiet K**, Stercz B, Lohinai Z, Dobay O. A bikarbonát és a pH szerepe a légutak védelmében - mire tanít minket a CF? In: Bagdy, György (eds.) FAMÉ 2019 Magyar Kísérletes és Klinikai Farmakológiai Társaság;

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