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Different conditioning agents provide TGF-β1 release from root dentin: an *in vitro* study

Diferentes agentes condicionantes promovem a liberação de TGF-β1 da dentina radicular: um estudo *in vitro*

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ABSTRACT

Objective: This study evaluated the effectiveness of different conditioning agents in promoting the release of Transforming Growth Factor Beta 1 (TGF-β1) from root dentin. **Material and Methods:** Sixty-eight third molars were distributed into four groups (n=17): 17% ethylenediaminetetraacetic acid (EDTA); 10% EDTA; 10% citric acid (CA); CA + 3% ferric chloride (10-3 solution). Slices of the cervical third of the root were obtained and irrigated with 2% chlorhexidine for 5 min. The slices were immersed in 220 μL of each conditioning agent for 10 min to expose TGF-β1 and analyzed by ELISA. Data normality was tested using the Shapiro-Wilk test. Kruskal-Wallis tests with Dunn-Bonferroni post hoc were used for comparison between groups ($p<0.05$). **Results:** Significant differences were observed among the treatments ($p<0.001$). The lowest TGF-β1 release was found in the group treated with 17% EDTA, which was significantly lower compared to the 10% EDTA, 10% CA and 10-3 solution groups. **Conclusion:** It could be concluded that different conditioning agents significantly affect the release of TGF-β1 from root dentin. Among the tested agents, 17% EDTA resulted in the lowest release, whereas 10% EDTA and the 10–3 solution promoted higher levels of growth factor release.

KEYWORDS

Dentin; Endodontics; Growth factor beta 1; Regeneration; Regenerative endodontics.

RESUMO

Objetivo: Este estudo avaliou a eficácia de diferentes agentes condicionantes na liberação de Fator de Crescimento Transformador Beta 1 (TGF-β1) da dentina radicular. **Material e Métodos:** Sessenta e oito terceiros molares foram distribuídos em quatro grupos (n=17): ácido etilenodiamino tetra-acético (EDTA) 17%; EDTA 10%; ácido cítrico (AC) 10%; e AC com cloreto férrico 3% (solução 10-3). Fatias do terço cervical da raiz foram obtidas e irrigadas com clorexidina 2% por 5 minutos. Em seguida, foram imersas em 220 μL de cada agente condicionante por 10 minutos para exposição do TGF-β1, e analisadas pelo ensaio ELISA. A normalidade dos dados foi avaliada pelo teste de Shapiro-Wilk. Para comparação entre os grupos, foram utilizados os testes de Kruskal-Wallis com pós-teste de Dunn-Bonferroni ($p<0,05$). **Resultados:** Diferenças significativas foram observadas entre os tratamentos ($p<0,001$). A menor liberação de TGF-β1 foi observada no grupo tratado com EDTA 17%, sendo significativamente inferior em comparação aos grupos tratados com EDTA a 10%, AC a 10% e com a solução 10-3. **Conclusão:** Pode-se concluir que diferentes agentes condicionantes afetam significativamente a liberação de TGF-β1 da dentina radicular. Entre os agentes testados, o EDTA 17% resultou na menor liberação, enquanto o EDTA 10% e a solução 10–3 promoveram níveis mais elevados de liberação do fator de crescimento.

PALAVRAS-CHAVE

Dentina; Endodontia; Fator de crescimento beta 1; Regeneração; Endodontia regenerativa.

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INTRODUCTION

Alternative approaches aimed at regenerating dental pulp have been gaining prominence in endodontics. Regenerative endodontic procedures are designed to restore the biological structure and original function of dental pulp, enhancing the root's resilience, length, and thickness compared to roots subjected to apexification [1]. The success of pulp regeneration relies on the presence of essential elements, including stem cells, scaffolds or support structures, and growth factors [2-4]. Among the growth factors, transforming growth factor beta 1 (TGF-β1) stands out due to its remarkable ability to recruit, to regulate cell proliferation, differentiation, and dentinogenesis, playing a crucial role in tissue repair and remodeling [5,6]. Furthermore, TGF-β1 demonstrates anti-inflammatory effects by regulating pro-inflammatory cytokines [7]. However, both TGF-β1 and other factors employed in the pulp regeneration process are confined within the dentin matrix, necessitating the use of dentin-conditioning agents for their release [7-9].

Conditioning agents, such as EDTA, in different concentrations and application times have been studied with the aim of increasing the release of bioactive molecules, such as TGF-β1, which are found within the dentin matrix. This phenomenon occurs during dentin demineralization, increasing migration, fixation and differentiation of stem cells from the periapical region [7-11]. Although EDTA is effective in removing the smear layer, dissolving organic material, and releasing growth factors, its prolonged use or use at high concentrations can cause detrimental effects on dentin. These effects include reduced microhardness, destruction of the dentin matrix, dentin erosions, and decreased fracture resistance, especially in immature teeth with thin dentin walls [12,13]. In view of the biological challenges in regenerative procedures, alternative conditioning agents to EDTA, such as citric acid (CA), have been investigated [14].

Research on the use of CA for dentin demineralization in pulp regeneration is justified in the search for more effective and less invasive dental treatments, highlighting its relatively mild action compared to other acids [15]. This agent, naturally found in citrus fruits such as lemons and oranges, can dissolve minerals like calcium in dentin. Being a weak acid, it can demineralize dentin in a controlled manner, increasing its permeability and facilitating pulp regeneration [16]. CA demonstrated the removal of the smear layer *in vitro*, exhibited lower cytotoxicity to fibroblasts, and showed greater biocompatibility compared to 17% EDTA [12,17]. Additionally, CA has been shown to be superior to EDTA concerning the recruitment, attachment, and survival of stem cells [18]. A systematic review reported that dentin conditioning with 10% CA promotes a greater release of TGF-β1 compared to EDTA, although both agents produced similar cellular responses [19]. Furthermore, a more recent systematic review noted methodological heterogeneity among studies but highlighted a tendency for increased TGF-β1 release with EDTA (10% or 17%) and with 10% CA [19]. Consequently, the researchers recommended conducting further studies with standardized protocols to validate these findings [19,20].

Considering research on new conditioning agents and the promising results observed in the dentin substrate using a solution containing 10% CA and 3% ferric chloride (10-3 solution) [21–24], we hypothesize that this combination could enhance the release of growth factors and, in the future, improve the regenerative endodontic procedures. Although the 10-3 solution has been investigated mainly in the context of adhesive dentistry to evaluate resin–dentin bonding, it has not yet been assessed in endodontic or regenerative procedures, representing an important gap in the literature. Some studies suggest that dentin conditioners containing iron may offer a protective effect on collagen. This assumption is supported by paleontological findings showing that iron contributes to the preservation of collagen and elastin in fossils [25–26]. Additionally, ferric chloride binds to collagen, providing protection against structural degradation and allowing for greater depth of dentin demineralization [24]. Therefore, it is assumed that a chelating agent such as CA promotes controlled demineralization of the dentin structure, while ferric chloride acts as a stabilizing agent, helping protect the dentin against degradation. Thus, the 10-3 solution may represent an interesting alternative for dentin conditioning.

Therefore, this study aims to evaluate the effectiveness of different conditioning agents on TGF-β1 release. The study hypothesis is that different conditioning agents result in varying amounts of TGF-β1 release. The study hypothesis

is that different conditioning agents result in variable rates of TGF-β1 release.

MATERIAL AND METHODS

Experimental design

The manuscript was prepared in accordance with the Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) guidelines [27]. Figure 1 shows the PRILE flowchart with the study steps. The sample consisted of 68 third molars randomly distributed in 4 experimental groups (n=17) considering the conditioning agents as follows: 17% EDTA; 10% EDTA; 10% CA; 10-3 solution. The sample size was calculated with a significance level of 0.05, effect size of 0.35 and power of 0.85, using the G* Power v3.1 program (Heinrich Heine, Universitat Dusseldorf, Germany). The dependent variable was the release of TGF-β1. The irrigating and conditioning agents, composition and manufacturers used in the study are described in the Table I.

Teeth selection and root dentin preparation

The local Research Ethics Committee approved this study (#64296022.8.0000.5418). Sixty-eight extracted permanent third molars, aged between 18 and 35 years, were donated. The specimens were free of carious lesions, with at least two roots and no pathological or anatomical

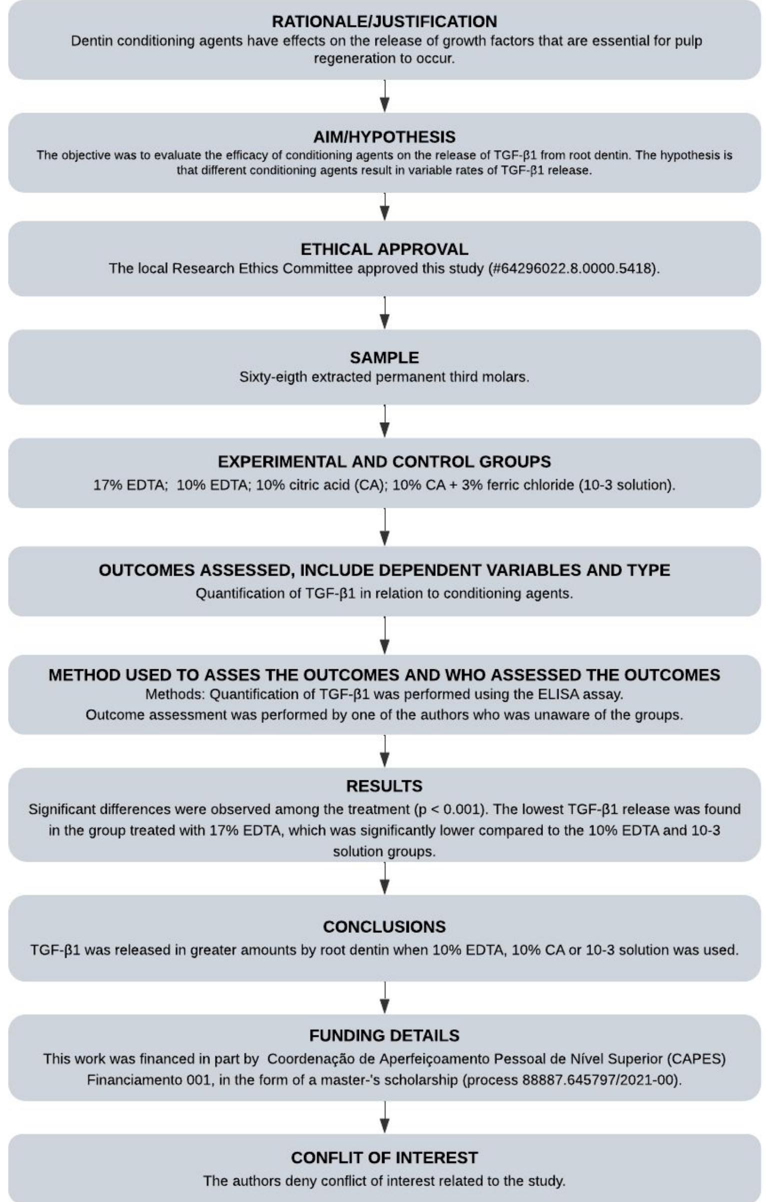

Figure 1 - Flowchart according to the Preferred Reporting Items for Laboratory Studies in Endodontology (PRILE) 2021 guidelines.

Table I - Composition and manufacturers of irrigant and conditioning agents

Irrigant Conditioning agents	Composition	Manufacturer Batch Number
2% Chlorhexidine	2% Chlorhexidine Digluconate Solution (pH=5.4) *	Biodinâmica # 0458/21
17% EDTA	17% EDTA (pH=7.0) *	Synth #205497
10% EDTA	10% EDTA (pH=7.0) *	Synth #205497
10% Citric Acid	10% Citric Acid (pH=3) *	Dinâmica #137943
10-3 Solution	10% Citric Acid[1] 3% ferric chloride[2] (pH=0.5) *	Dinâmica [1]#137943 [2]#25449

*The pH values of the solutions were determined using a pH indicator paper strip for turbid solutions MERCK McolorpHast™, São Paulo/Brazil

changes. With the help of periodontal curettes, bone, and periodontal tissue, when present, were removed. They were subsequently frozen at -80°C in 0.9% saline solution.

The teeth were sectioned in a perpendicular to the long axis, at the cementoenamel junction, using a straight handpiece and a water-cooled low-speed diamond disc. The roots were measured with a digital caliper (Mitutoyo, Suzano, SP, Brazil) and the crowns were discarded. The first cervical third was sectioned in a similar way, the remaining roots were discarded, and specimens of 3 mm thick root dentin were obtained. After that, the exposed dentin surface was polished with 600 silicon carbide grit to expose the pulp in occlusal and apical views. The remaining pulp was removed, and the specimens were stored in 0.5% chloramine for 24 h at 4°C and before the experiment, the solution was replaced with deionized water [28].

Radicular dentin treatment

The specimens were externally coated with an acid-resistant varnish and treated with 2 mL of 2% CHX irrigating solution for 5 min [11,28]. After the irrigation solution was used, the specimens were transferred to a 48-well plate and treated with 220 μL (protocol determined in a pilot study, unpublished data) of 17% EDTA (pH=7), 10% EDTA (pH=7), 10% CA (pH=3), and 10-3 solution (pH=0.5) as conditioning agents, for 10 min, on a shaker table (CT-165, Cientec, Belo Horizonte, MG, Brazil) to expose dentin growth factors. The samples were shaken to simulate the clinical dynamics of irrigation that occur during the chemical preparation of the root canal. This volume (220 μL), is sufficient to fully immerse the specimen, ensuring complete contact with the conditioning solution [11,28]. The solutions applied for each specimen after irrigation were stored and frozen for subsequent assessment and TGF-β1 quantification.

Quantification of TGF-β1 by ELISA assay

TGF-β1 levels were quantified using the Quantikine® ELISA kit (DuoSet Human TGF-b1, R&D Systems, Minneapolis, MN, USA, #P323635) specific for human, mouse, rat, porcine, and canine TGF-β1. This is a solid-phase sandwich enzyme immunoassay that employs monoclonal and polyclonal antibodies specific for TGF-β1. Samples were pre-activated to convert latent TGF-β1 into its immunoreactive form. For this, 100 μL of sample were acidified with 20 μL of 1 N HCl and incubated for 10 min at room temperature. Then, the samples were neutralized with 20 μL of 1.2 N NaOH/0.5 M HEPES solution. After neutralization, the activated samples were diluted according to the manufacturer's instructions using Calibrator Diluent RD6-11 (prediluted 1:2), and the final dilution factor was considered for data analysis. Optical density was measured at 450 nm with wavelength correction at 540 nm using a microplate reader. TGF-β1 concentrations were calculated by interpolation from a seven-point standard curve (31.3 to 2000 pg/mL) using a four-parameter logistic (4-PL) model and corrected for the dilution factor.

Statistical analysis

Data normality was tested using the Shapiro-Wilk test. Kruskal-Wallis tests with Dunn-Bonferroni post hoc were used for comparison between groups. P values with Bonferroni correction <0.05 were considered statistically significant. Statistical analyses were performed using JASP for Windows software (JASP Team, Version 0.19.1) and graphs were made using GraphPad Prism 8.0 for Windows (GraphPad Soft-ware, Boston, Massachusetts USA).

RESULTS

The results for the release of TGF- β1 were presented with statistic (H), p value (p) and effect size (Eta-squared) for Kruskall-Wallis, and statistic (z), p value considering Bonferroni correction (p_{bonf}) and Rank-biserial correlation (r_{rb}).

The Kruskal-Wallis test showed that there was a statistical difference in the release of TGF-β1 ($H(3)=20.844$; $p<0.001$; rank $h^2=0.279$). In the comparison between groups, considering the Bonferroni correction, the 17% EDTA group showed lower values in release when compared to the 10% EDTA group ($z=3.202$; $p_{bonf}=0.008$; $r_{rb}=0.661$) and the 10-3 solution ($z=4.416$; $p_{bonf}<0.001$; $r_{rb}= 0.768$). There was no statistical difference when comparing the 10% EDTA group with the 10-3 solution group ($z=1.215$; $p_{bonf}=1.000$; $r_{rb}=0.298$), and furthermore, the CA group did not show a statistical difference with 10% EDTA ($z=2.412$; $p_{bonf} = 0.095$; $r_{rb} = 0.571$), 10% EDTA ($z=-0.790$; $p_{bonf} = 1.000$; $r_{rb} = 0.190$), and 10-3 Solution ($z=2.004$; $p_{bonf}=0.270$; $r_{rb}=0.457$). Table II shows the descriptive data of the groups considering the results in pg/mL.

DISCUSSION

Regenerative therapies rely on three core elements: stem cells, structures, and growth factors. However, achieving comprehensive success in regeneration requires the essential incorporation of materials, irrigants, conditioners, and medications throughout the regenerative endodontics procedures. Effectively addressing all pertinent aspects and variables related to regenerative success in young permanent teeth is a remarkable task [3,4].

Considering the importance of understanding the impact of agents used in regenerative endodontic procedures on growth factor release, the hypothesis of this study was confirmed, since significant differences were observed between root dentin conditioning agents. In the present study, we investigated 10% and 17% EDTA in 10 min, a shorter period compared to previous studies [11,28]. In addition, we evaluated two additional conditioning agents, 10% CA and a combination of CA with ferric chloride (10-3 solution). All of these conditioning agents provided TGF-β1 release.

We observed greater release of TGF-β1 when 10% EDTA was used compared to 17% EDTA. This finding supports the observations made by Galler et al. [11], who reported that conditioning with 10% EDTA resulted in the greatest release of TGF-β1, whereas 17% EDTA was less effective. In addition, the results of the present study indicated that 17% EDTA had the lowest levels of growth factor release when compared to 10% EDTA (pbonf=0.008) and 10-3 solution (pbonf<0.001), with strong effect sizes (rrb=0.661 and 0.768, respectively). This can be explained by considering the impact of EDTA on dentin. When the EDTA concentration increases, its osmolarity also increases, probably causing an acid accumulation during the exchange process of hydrogen and calcium ions present in hydroxyapatite. This higher concentration of acid can directly influence the EDTA action into calcium ions of root dentin and result in unwanted effects, such as dehydration of dentin and changes in the physical properties of cleaning solutions [29]. These findings highlight the importance of considering not only the demineralizing capacity of conditioning solutions but also their effects on the dentin matrix and the release of growth factors essential for regeneration.

Table II - Descriptive and statistical comparisons among groups (pg/mL)

	10-3 Solution [b]	Citric Acid [ab]	10% EDTA [b]	17% EDTA [a]
Median	65.050	22.190	38.300	4.250
Minimum	1.750	2.900	1.750	0.000
Maximum	342.500	70.820	151.520	31.130

Different lowercase letters mean statistical difference between the groups (p<0.05).

In this context, CA especially when combined with ferric chloride, in our study, has proven promising, exhibiting a TGF-β1 release rate notably superior to that achieved with 17% EDTA. This combination stands out for its impressive results and the potential application of CA, given its proven non-cytotoxic nature *in vitro* [12]. These findings reinforce the assumption that CA is effective in TGF-β1 release, aligning consistently with results from comparable *in vitro* studies reported in two recent systematic reviews [19,20].

Furthermore, the release of TGF-β1 in the group subjected to CA as a conditioning agent showed superior results compared to that treated with 17% EDTA and comparable results to that treated with 10% EDTA. These findings align with studies, which similarly compared 17% EDTA and CA [2,18]. The consistent outcomes underscore that etching with CA attracts a significantly higher number of stem cells to dentin when compared to EDTA [17]. Therefore, conditioning with CA may be a viable alternative to current treatments, along with favorable biocompatibility in the context of regenerative endodontics.

Conditioning agents play an important role in regenerative endodontics by facilitating root surface cleaning and creating a substrate that promotes cell adhesion and migration, both essential for the regenerative process [11]. Additionally, many of these agents contain acids that can demineralize root dentin, thereby opening dentinal tubules and enhancing the penetration and migration of stem cells and other regenerative components. In our quest for novel conditioning agents aligning with these objectives, we have explored combinations displaying promise. In this context, we studied the combination of CA with ferric chloride in the present study. Rodrigues et al. [24] previously investigated this combination, aiming to assess the effects of conditioning solutions containing ferric chloride ($FeCl_3$) on the resin-dentin bond strength, the safeguarding of dentin collagen against enzymatic degradation, and the activity of cathepsin-K [24]. Their study demonstrated that $FeCl_3$ binds to collagen, providing protection against cathepsin K-mediated degradation. This supports the notion that the combination of citric acid and ferric chloride represents a favorable alternative for dentin conditioning, with the added advantage of preventing collagen degradation [24].

The 10–3 solution has emerged as a versatile and effective alternative for dentin conditioning. Saeki et al. [30] demonstrated that the addition of ferric chloride enhances the demineralizing effect of CA, intensifying dentin conditioning without causing irreversible collapse of the collagen matrix [30]. More recent studies indicate that the 10–3 solution can be a viable alternative to phosphoric acid, even on dry substrates, providing a stable adhesive interface without compromising the mechanical properties of dentin [31]. Additionally, the literature shows that this solution is highly effective in removing the smear layer without causing excessive demineralization, favoring controlled exposure of dentinal tubules and the release of bioactive signals important in regenerative endodontic procedures [32]. Its effectiveness even in the presence of blood contamination reinforces its clinical applicability in challenging scenarios such as reimplantation and regenerative therapies, promoting both durable adhesion and a microenvironment conducive to tissue regeneration [33]. Based on this assumption, one of the objectives of the present study was to investigate whether the favorable effects observed with $FeCl_3$ on the bond strength between dentin and resin could potentially extend to one of the factors involved in the regenerative processes. Our findings indicated that the 10-3 solution significantly enhanced the release of TGF-β1 from root dentin, exceeding even the levels achieved with 17% EDTA. These results suggest that further exploration of this combination is warranted, especially concerning its implications for various procedures involving root dentin.

Although this study provides relevant contributions, some methodological limitations should be acknowledged. The *in vitro* design does not fully replicate the complex biological conditions of the clinical environment, particularly regarding the interaction of conditioning agents with vital tissues, inflammatory mediators, and blood components. Another limitation is the absence of a negative control group without treatment, which could have strengthened the interpretation of the results. However, it is important to note that a negative control would not be applicable to the clinical scenario, because even if no release occurred, or if it were lower than that of the test groups, such a condition could not be used in clinical practice, given the additional characteristics that a conditioning agent must present. Considering these limitations, future studies should incorporate biologically relevant models to evaluate stem cell viability, dentin surface alterations, and the clinical applicability of these conditioning protocols.

It is important to highlight that the use of conditioning agents during pulp regeneration protocols may affect the structure of root dentin, thereby influencing the release of growth factors, particularly TGF-β1. Our findings reinforce the importance of continually investigating the conditioning agents currently used, as other available solutions may also yield favorable outcomes. The selection of a conditioning agent for pulp regeneration should consider not only its antibacterial efficacy during root canal disinfection but also its effects on stem cells and on the release of dentin-derived growth factors. In addition to the present results, further studies are necessary to deepen the understanding of how different conditioning agents influence the regenerative potential of root dentin.

CONCLUSION

This study demonstrated that different conditioning agents significantly affect the release of TGF-β1 from root dentin. Among the tested agents, 17% EDTA resulted in the lowest release, whereas 10% EDTA and the 10–3 solution promoted higher levels of growth factor release.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author's Contributions

ALT: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft Writing - Review & Editing. CMBG: Methodology, Investigation, Formal analysis, Visualization, Writing - original draft Writing - Review & Editing. VABS: Methodology, Investigation, Formal analysis, Writing - original draft, Writing - Review & Editing. RMPR: Conceptualization, Investigation, Formal analysis, Writing - Review & Editing. FMP: Conceptualization, Investigation, Formal analysis, Supervision, Writing - original draft, Writing - Review & Editing

Conflict of Interest

The authors deny any conflicts of interest related to this study.

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Regulatory Statement

This study was conducted in accordance with all the provisions of the local human subjects oversight committee guidelines and policies of the Faculdade de Odontologia de Piracicaba (FOP), University of Campinas (UNICAMP). This study protocol was reviewed and approved by the local Research Ethics Committee, approval number 64296022.8.0000.5418.

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