http://doi.org/10.33279/jkcd.v14i04.815

Orginal Article

ISOLATION OF HUMAN ADULT DENTAL PULP STEM CELLS USING ENZYMATIC DIGESTION

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ABSTRACT

Objectives: *The aim of the study was to culture dental pulp stem cells from non- carious, extracted human adult permanent teeth using enzymatic digestion*

Materials and Methods: *Human adult extracted teeth were collected from the Oral Surgery Department OPD, from the Ziauddin Dental College. Pulp was extirpated via barbed broach and isolated via enzymatic digestion using trypsin. The stem cells were cultured under conditioned media and a growth curve was plotted against the population doubling time. Cell viability was tested and finally the cells were cryopreserved for future use. Data was presented descriptively by graphical representation.*

Results: *The stem cells exhibited spindle shaped morphology similar to mesenchymal stem cells after two weeks of isolation. The cells reached 80% confluency after a total of four weeks after which before they were processed for further assays. The growth characteristics were analyzed by calculation of population doubling time and cell viability.*

Conclusion: *In the study successfully demonstrated the ability to isolate and culture dental pulp stem cells (DPSCs) from non-carious, extracted human adult permanent teeth using an enzymatic digestion method. The enzymatic digestion method of harvesting stem cell resulted in a stable DPSC cell growth. This method is convenient and a reliable source of stem cells for future personalized medicine research.*

Key words: *Dental Pulp Stem Cells, Regenerative medicine, Enzymatic digestion method, Cell culture*

Cite as: Khan S, But S, Usman S, Mirza S. Isolation of human adult dental pulp stem cells using enzymatic digestion. Journal of Khyber College of Dentistry Dec 2024, Vol. 14, No. 4. http://doi.org/10.33279/jkcd.v14i04.815

INTRODUCTION

Dental pulp stem cells (DPSCs) are a type of mesenchymal stem cells (MSCs) residing within the dental pulp tissue of teeth. Dental pulp stem cells were initially isolated by Gronthos et al in 2000. Since then, many researchers have identified these stem cells for their remarkable properties. These cells are known for their self-renewal capacity and immunomodulatory properties, making them promising candidates for regenerative therapies[1].

A range of mesenchymal stem cells (MSCs) have been successfully isolated and characterized from various dental tissues, including dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), dental follicle progenitor cells (DFPCs), stem cells from human exfoliated deciduous teeth (SHED), apical papillary stem cells (SCAP), and gingival mesenchymal stem cells (GMSCs)[2]. SCAP are derived from the apical papilla found at the apex of the root of a developing tooth. They show a greater potential to regenerate dentine as comparted to dental pulp stem cells. PDLSCs originate from the soft con-

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Date Submitted: June 2024
Date Revised: August 2024
Date Accepted: September 2024

nective tissue, the periodontal ligament, and exhibit a tendon specific transcription factor as compared to DPSCs, therefore are integral to the regeneration of periodontal tissues. DFPCs are derived from the connective tissue surrounding the developing tooth bud and exhibit a higher proliferative potential as compared to DPSCs[3].

Dental pulp stem cells originate from the neural crest and express mesenchymal and neural stem markers. They exhibit multipotency, meaning they can differentiate into various cell types. These cells exhibit trilineage differentiation forming adipocytes, chondrocytes and osteocytes. Furthermore, they can differentiate into odontoblasts, neurogenic, musculoskeletal and angiogenic lineages[4]. Therefore, DPSCs can potentially be used for cartilage regeneration[5], peripheral nerve repair[6], formation of dentine pulp complex[7], and trans differentiation into neuron like cells.

DPSCs have become an increasingly popular source of stem cells due to their accessibility, ease of collection, economic benefit and lack of ethical concerns. Tooth extraction, a routine surgical procedure typically conducted under local anesthesia, can be undertaken at any stage of adulthood. Normally, the extracted tooth is considered biological waste. The primary source for harvesting dental pulp stem cells (DPSC) is the pulp tissues of human wisdom teeth or premolars extracted for orthodontic purpose. Although less common, supernumerary teeth, mesiodents, or third premolars may also be utilized. Third molars are commonly extracted to prevent recurrent inflammation of the soft tissues surrounding the partially erupted tooth. Other sources of DPSC are the stem cells from human exfoliated deciduous teeth (SHED) and stem cells from apical papilla (SCAP). However, wisdom teeth and premolars extracted due to orthodontic reasons are more reliable sources of obtaining DPSCs[8].

There are two methods for harvesting DPSCs, the explant or outgrowth method and the enzymatic digestion method. The explant method involves the cultivation of small, undigested pieces of dental pulp tissue on a culture dish. The cells migrate or outgrow from the tissue explants into the surrounding culture medium over time. This method is simple and does not require the use of enzymes, which may preserve the natural cell microenvironment. However, it is generally a slower process and may result in a lower yield of isolated cells compared to the enzymatic digestion method[9]. In contrast, the enzymatic digestion method involves the use of enzymes to break down the extracellular matrix and dissociate cells from the tissue. The dental pulp is typically incubated with enzymes such as collagenase and dispase, which degrade the collagen and other components of the extracellular matrix, thereby allowing the cells to be isolated more efficiently[10]. This method usually results in a higher yield of DPSCs in a shorter period, making it the preferred choice for many studies focusing on stem cell isolation. However, enzyme choice and concentration must be carefully optimized to avoid damaging the cells.

In this study, we chose to use trypsin as the enzyme for the digestion process. Trypsin is a well-known proteolytic enzyme that works by cleaving peptide bonds in proteins, facilitating the dissociation of cells from the tissue matrix. Compared to collagenase and dispase, trypsin provided more effective and efficient cell dissociation in our experiments, resulting in a higher yield of viable DPSCs. Furthermore, trypsin is generally more cost-effective than collagenase and dispase, making it an attractive option for large-scale isolation of stem cells, particularly in research settings where cost considerations are important[11].

The present study was intended to establish a protocol using the enzyme trypsin for isolation and cryopreservation of DPSCs for a long-term storage to allow experiments and assays using both in vitro and in vivo approaches for their use in regenerative medicine.

MATERIALS AND METHODS

This in-vitro study was approved by the ethical review board of Ziauddin University (Reference code: 5760722SKOM). All human participation was voluntary and as per the Helsinki Declaration. Each participant provided a written informed consent. A total of 12 patients were recruited from the Oral Surgery department Dental OPD at Ziauddin Dental College (Table 1). Healthy adults aged between 18-55 years of either sex and referred for extraction of a premolar for orthodontic reasons or wisdom tooth were included whereas individuals with systemic illness, periodontal disease, dental caries, pulpitis or radiographic evidence of periapical periodontitis

of endodontic origin were excluded from the study.

A total of 12 dental pulp samples from these patients were included in the study. However, not all of them yielded viable DPSCs due to contamination issues. The samples were cultured individually, and only those with successful cell harvests were used for the study.

The patients were asked to rinse with 0.2% chlorhexidine before they were anesthetized with local anesthesia 2% lidocaine. Non-surgical extractions were performed under sterile conditions and the extracted sample was collected in chilled transport media (PBS+DMEM) as shown in Fig.2.

The collected sample was then washed thoroughly with 1xPBS.

A sterilized handpiece (NSK, Japan) was used with a straight fissure diamond bur (Mani, Japan) to carry out the chamber opening using copious amount of saline. After gaining access into the pulp chamber and deroofing, a #15 barbed broach (Mani, Japan) was used to extirpate the pulp from the canal and then the pulp along-with the barbed broach was transported to a 15ml falcon tube with the pulp tethered around it (Figure 1). The tube was transferred to the Ziauddin cell culture lab under the Biosafety Level 2 (VRM, USA). The pulp was gently removed from the barbed broach and washed thoroughly with PBS in a petri dish. The pulp was then minced with a no. 15 surgical blade followed by addition of 10x trypsin (concentered trypsin 5g/L and 2g EDTA salt) for tissue digestion into a falcon tube (Figure 3). The tube was placed in an incubator (Esco cell culture CO2 incubator, Singapore) for 10 minutes to optimize the function of trypsin and then centrifuged for 8 minutes at 11,186 g at room temperature. Finally, the fragmented dental pulp was seeded in a T-25 flask with conditioned media (DMEM, 4Mm L-glutamine, 10% FBS, 1%penicillin/streptomycin, 1Mm sodium pyruvate. The T-25 flask was then cultured in 37 ^{0}C, 5% CO2 incubator (Esco, Singapore) and with media changes every 3rd day.

The cells were observed under a Floid cell imaging microscope (Invitrogen, USA) and morphology captured using the microscope camera (Floid cell imaging station software). Furthermore, differential interference contrast (DIC) microscopy (Nikon, Japan) was used to observe cells at p0 and p1. Once the cells reached 80% confluency, they were sub-passaged for further experiments and cryopreservation. DPSCs were washed with PBS followed by addition of 0.25% trypsin. The flask was left in the incubator for 5 minutes and then enzyme action was ceased by the addition of conditioned media. Detached cells were transferred to a 15ml falcon tube and run in the centrifuge at 10,000RPM for 8 minutes. This allowed the cell pellet to settle at the bottom and the supernatant was easily removed. The cells were then resuspended into media and seeded into a T-25 and T-75 flask. One falcon tube was used for cryopreservation, the cryomedia (95% FBS and 5% DMSO) was directly added to the cell pellet and placed in -20°C freezer for two hours, then -80°C freezer for 24 hours and finally submerged into -196°C liquid nitrogen tank and stored for future use.

POPULATION DOUBLING TIME

Population doubling time was calculated after every 6 days at different passages.

PDT is calculated as:

DT= T ln2/ln (Xe/Xb)

DT= Doubling time

T= Incubation time

Xb= Cell number at beginning of incubation time

Xe= Cell number at end of incubation time

After trypsinization and cell resuspension 0.4% trypan blue was mixed to the prepared sample for cell viability analysis. The cells were loaded onto a hemocytometer chamber and a total cell count was done.

The formula used for cell viability was:

% Viable cells= [1- (number of dead cells/total number of cells)] x 100

Data was presented descriptively by graphical representation.

RESULT

The isolated cells grew in colonies forming clusters, the cells expressed fibroblast like spindle-shaped morphology. The cells were rapid growing and reached 80% confluency by the end of the 3rd week, as shown in the results in Figures 4(a) and 4(b).

The cells were observed everyday under live

cell imaging inverted microscope (Invitrogen, USA).

The cells were also observed under a differential inverted compound microscope (Nikon type 120c, Japan) at x20 magnification as seen in Figure 4.

The population doubling time is the time needed for a culture to double in number.

Population doubling time growth curves are plotted on a graph as observed in Figure 6a, 6b,

6c and 6d for passage 1, 2, 3 and 4 respectively. The population doubling time after 6 days was 24.57hours at passage 1, 22.63hours at passage 2, 23.10hours at passage 3 and 23.10hours at passage 4. The cells observed exhibited a high proliferation rate and there was no significant different between the population doubling time at different passages.

Cell viability culture data is presented in figure no 5. Cell viability was tested using trypan blue exclusion method to check for the integrity of the cell. Cell viability was plotted using a bar chart.

Table 1: Data for sample collection

Tooth indicated for extraction	Age (Y)	Gender
Third molar	45	F
Third molar	38	F
Painful third molar	29	M
Non impacted third molar	25	F
Third molar	40	M
Third molar	43	F
Third molar	52	M
First premolar indicated for orthodontic extraction	16	M
Second premolar indicated for orthodontic extraction	18	M
Second premolar indicated for orthodontic extraction	20	F
Second premolar indicated for orthodontic extraction	13	F
First premolar indicated for orthodontic extraction	14	M

Table 2: Population Doubling Time

Dental Pulp Stem Cells	Cell count	PDT
P1	150x104	24.57hrs
P2	189x104	22.63hrs
P3	178x104	23.10hrs
P4	177x104	23.10hrs

Cell viability was not compromised at the different passages; however, cell death is observed when cells become overconfluent and there is no space for more cells to grow resulting in cell death.

DISCUSSION

The aim of this study was to isolate dental pulp stem cells from the pulp tissue using enzymatic digestion method. Enzymatic digestion is a widely used method for isolating stem cells from various tissues due to its efficiency and reliability[12]. However, it's essential to optimize the isolation protocol to ensure maximum cell yield and viability while minimizing damage to the cells. Additionally, researchers continually explore alternative methods for stem cell isolation to improve efficiency and reduce the risk of contamination or damage to the cells[10].

Recently, several isolation protocols have been established to extract dental pulp stem cells. A study by Raoof et al compared three different isolation methods; explant by outgrowth, digestion of pulp by enzyme and fixing digested pulp under a coverslip in the medium. The enzyme digestion method exhibited a higher proliferation rate. In this particular study we established primary cell culture using 10X trypsin for digestion of pulp tissue for maximum yield. 10x trypsin allowed a higher yield in tissue dissociation

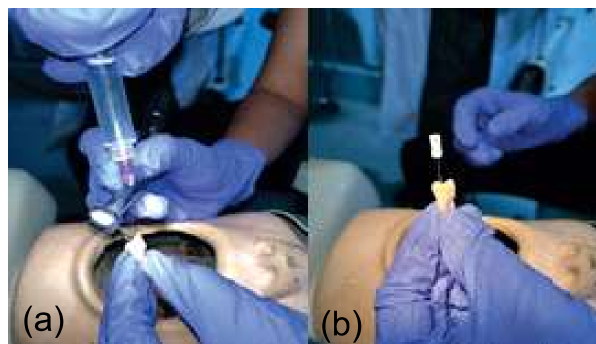

Fig 1: Photograph showing chamber opening using handpiece under copious irrigation with saline (a), followed by extirpation of pulp using a #15 barbed broach (b).

The decision to use an access cavity and a barbed broach for pulp extirpation rather than using a chisel to section the tooth is to prevent contamination with controlled removal of pulp versus using a chisel against which may introduce a non-sterile environment. The chisel can cause crushing therefore causing trauma to the tooth and compromising cell quality.

Fig 2: Pulp collected from extracted tooth and transported to the lab in transport media in a 15ml falcon tube.

Fig 3: Pulp thoroughly washed with 1x PBS (Phosphate buffer saline) in a petri dish. The pulp is then minced in the petri dish using a no. 15 blade, the pulp minced pulp tissue pieces are then digested using trypsin enzyme.

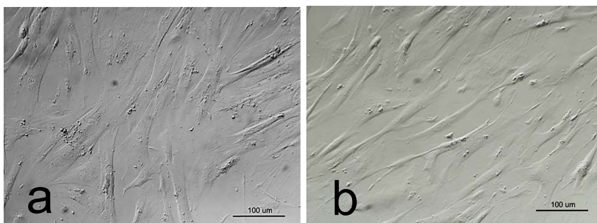

Fig 4: 4(a) Dental pulp stem cells observed at 20X magnification under TE 2000 Nikon (Japan) Differential Interference Contrast Microscope at different brightness contrast. The cells observed here are at stage P0. (Scale bar =100 µm) 4(b) Cells were observed at 20X magnification at stage P1. (Scale bar =100 µm)

without damaging the dental pulp stem cells. A review by Rodas-Juno 2017[13] discusses the different enzyme digestion protocols using different concentrations of collagenase, dispase, trypsin and acutase. However, there has been no comparison of digestion protocols between them, so the ideal conditions for obtaining more viable cells have not been assessed.

The enzyme digestion method is often preferred over explant techniques for isolating dental pulp stem cells (DPSCs) due to several advantages. Recent studies highlight the advantages of the enzyme

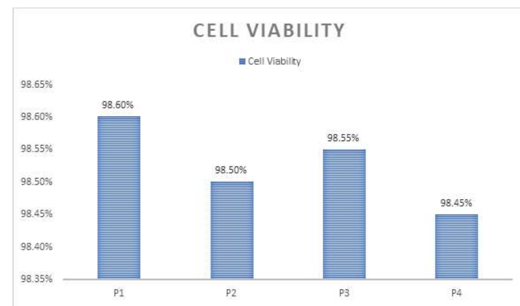

Fig 5: Cell viability across passages. A bar chart illustrates the percentage of viable cells at different passages. Cells at passage 1 were 98.60% viable, passage 2 were 98.50% viable, passage 3 were 98.55% viable and at passage 4, 98.45% cells were viable.

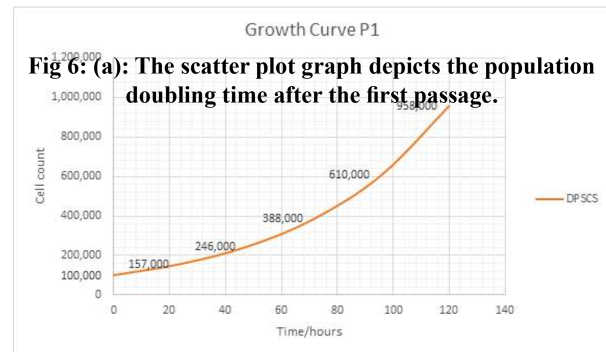

Fig 6: (a): The scatter plot graph depicts the population doubling time after the first passage.

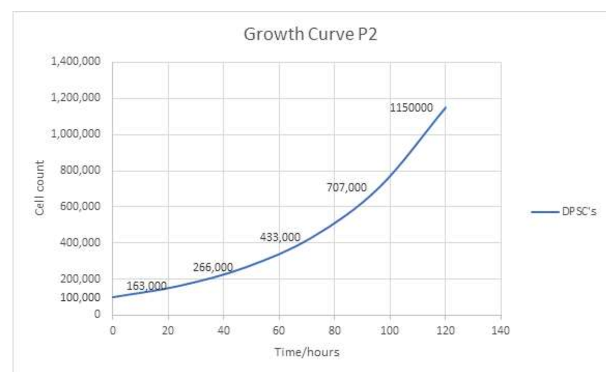

Fig 6: (b): This scatter plot graph depicts the growth curve after cells have been sub cultured and at passage 2.

digestion method in terms of efficiency, yield, and cell quality, making it a preferable choice in the isolation of dental pulp stem cells. Enzymatic digestion typically results in a greater number of viable DPSCs compared to explant methods. This is because enzymes like collagenase and dispase break down the extracellular matrix, allowing for the release of a higher density of stem cells from the tissue[14]. Enzyme digestion generally reduces the time needed for cell isolation. While explant techniques can take several days for cells to migrate out of the tissue, enzymatic methods can yield isolated cells within a few hours, making the process more efficient. Due to quick processing time the stress on cells is minimized, preserving stemness and differential potential. Therefore, cells isolated via enzyme digestion often demonstrate better viability and functionality[15].

There is currently no established gold standard practice for processing dental pulp tissue. In this study we used the recommended protocol using a diamond bur to expose the pulp chamber and a barbed broach to extirpate the pulp. A recent study by Kearney et al 2024[16] described a method used to reduce mechanical trauma by exposing pulp chamber without direct contact with pulp tissue. A water-cooled circular saw was used to separate the tooth at the cemento-enamel junction to incubate the pulp tissue in a coated flask using the explant outgrowth method. This technique should optimize cell growth and attachment while minimizing the risk of cell damage.

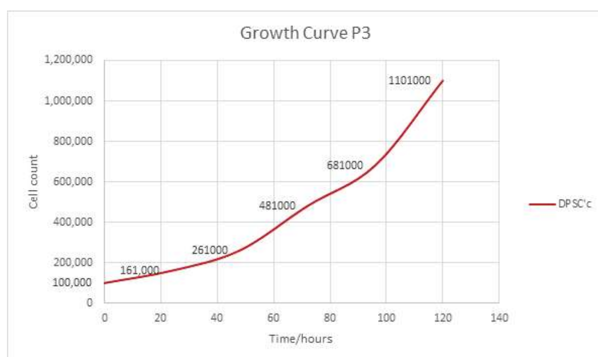

Fig 6: (c): Population doubling growth curve after being sub-cultured and taken to the third passage.

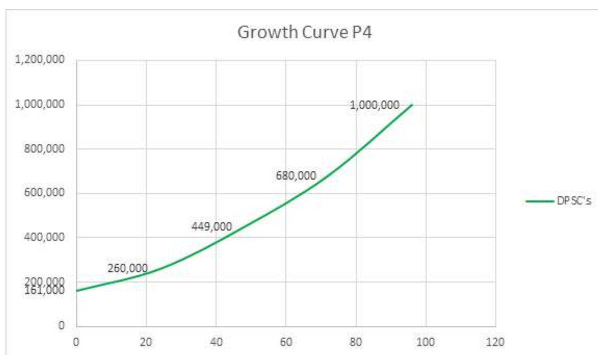

Fig 6: (d): Similarly, this graph depicts cells at passage 4.

The cells that emerged after isolation from the digested tissue pieces exhibited fibroblast like spindle shaped morphology with elongated cytoplasmic extensions. The stem cells formed monolayers and small clusters of cells closely adhering to one another. Advances in cell culture techniques have refined the characterization of these cells, showing more precise variations in shape and size based on culture conditions and passage number[17].

Dental pulp stem cells, due to their multilineage potential play a vital role in regenerative medicine. After successful isolation of dental stem cells various molecular mechanisms can affect the differentiation process toward chondrogenesis dentinogenesis, osteogenesis, adipogenesis and neurogenesis[4]. DPSCs have shown promising results in regeneration of bone and dental tissues. Recent researchers have demonstrated their ability to differentiate into osteoblast like cells critical for dental tissue regeneration[18]. Due to their neural crest origin these cells can contribute to neuroregeneration as well with studies suggesting their role in treating neurodegenerative conditions[19]. Dental pulp stem cells have also been explored for their potential in soft tissue repair, including skin regeneration and muscle repair[20].

Therefore, due to their biological characteristics and their potential use in regenerative medicine, dental pulp stem cells play a key role in cellular therapy. Therapeutic applications of dental stem cells are a rapidly evolving field with research spanning in various areas. Recent studies highlight the diverse applications of DPSCs in regenerative medicine and provide a foundation for understanding their therapeutic potential. A review by Morsczeck C. et al 2022[21] represents the application of dental stem cells in the treatment of dental caries and pulp necrosis. Another recent article explores the bone regenerative potential crucial for bone graft and repairs[22]. One of the most recent ongoing types of research regarding DPSC's is the potential treatments for neurological disorders through differentiation into neuronal cells[23]. Furthermore, due to lack of current

treatment modalities for neurodegenerative diseases the neurogenic potential of dental stem cells is yet to be investigated.

A limitation of this study may be its methodology. The effectiveness of enzymatic digestion can vary depending on the specific enzymes used. Inappropriate enzyme concentrations or activity levels may lead to incomplete digestion or damage to the cells. Similarly, prolonged treatment with the enzyme can affect the expression of surface markers and over-digestion can lead to cell damage or raise cell viability concerns.

On the basis of the results of this study we propose future studies that can further enhance the isolation protocol of the stem cells in general and DPSCs in particular. The isolation protocol of dental pulp stem cells plays a pivotal role in unlocking their therapeutic potential. By meticulously extracting and characterizing these multipotent cells, researchers and clinicians can harness their regenerative abilities to advance the fields of dentistry, orthopedics, neurology, and beyond. As isolation techniques continue to evolve, so will our ability to harness the transformative power of dental pulp stem cells in regenerative medicine.

CONCLUSION

In conclusion, our study successfully demonstrated the ability to isolate and culture dental pulp stem cells (DPSCs) from non-carious, extracted human adult permanent teeth using an enzymatic digestion method. The enzymatic digestion method of harvesting stem cell resulted in a stable DPSC cell growth. This method is convenient and a reliable source of stem cells for future personalized medicine research. The high proliferative capacity and multilineage differentiation potential of these cells underscore their promise as a valuable resource for cell-based regenerative therapies. These findings contribute to the growing body of evidence supporting the use of DPSCs in various therapeutic applications, particularly in tissue engineering and regenerative medicine. Further investigations are needed to optimize culture conditions and fully characterize the regenerative capabilities of DPSCs in vivo.

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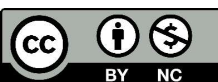
CONFLICT OF INTEREST
Authors declare no conflict of interest.
GRANT SUPPORT AND FINANCIAL DISCLOSURE
None declared.

AUTHORS' CONTRIBUTION

The following authors have made substantial contributions to the manuscript as under:

Conception or Design:	SK, SB, SU, SM
Acquisition, Analysis or Interpretation of Data:	SK, SB, SU, SM
Manuscript Writing & Approval:	SK, SB, SU, SM

All the authors agree 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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