Mesoporous Silica Nanoparticles Rescue H$_2$O$_2$-induced Inhibition of Cardiac Differentiation

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ABSTRACT. The anti-oxidative property of mesoporous silica nanoparticles (MSNs) has been proposed previously, which prompted us to investigate the potential protective effect of MSNs on human embryonic stem cells (hESCs) against oxidative stress. To this purpose, the cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assay. Apoptosis was analyzed by Annexin V/propidium iodide double-staining method. The intracellular glutathione, superoxide dismutase and malondialdehyde were measured with commercial assay kits. The reactive oxygen species was detected by staining with fluorescent dye DCFH-DA. The relative levels of Nkx2.5, Mef2c, Tbx5, dHand and α-MHC transcripts were measured by real-time polymerase chain reaction. The protein levels of Connexin 43, Troponin C1 and GAPDH were determined by immunoblotting. The beating behavior of embryoid bodies (EBs) was visually examined. Our results demonstrated that MSNs reversed hydrogen peroxide (H$_2$O$_2$)-inhibited cell viability and ameliorated H$_2$O$_2$-induced cell apoptosis in vitro. The H$_2$O$_2$-elicited intracellular oxidative stress was significantly relieved in the presence of MSNs. Furthermore, MSNs improved H$_2$O$_2$-suppressed differentiation of hESC-derived EBs and the maturation of the cardiomyocytes. In addition, MSNs treatment enhanced the beating properties of EBs. MSNs effectively conferred protection on hESCs against oxidative stress with respect to cardiac differentiation.

Key words: Mesoporous silica nanoparticles, hydrogen peroxide, human embryonic stem cells, differentiation

Introduction

Nanotechnology has been widely applied in biomedicine and attracts increasing attentions in biomedical research (Heath, 2015). Accumulating investigations have demonstrated the promising application prospects of nanotechnologies in diabetic therapeutics (Prow et al., 2008), tissue engineering (Shi et al., 2010) and cancer therapeutics/diagnosis (Janib et al., 2010). The crucial developments in nanotechnology and drug delivery system have surmounted many common obstacles in conventional medicine, and shown great advantage over traditional drugs in targeting and bioavailability (Mitragotri et al., 2015). Meanwhile, the novel system significantly improves drug stability and release dynamics, and consequently maximizes the therapeutic effects and minimizes side effects (Pryzhkova, 2013). Therefore, exploitations of novel and diversified nanodrug delivery systems have become the critical subject in nano-biomedicine. Currently, the regular and mainstream drug carriers include liposome, macromolecular polymer, Au nanoparticle, quantum dot and mesoporous silica nanoparticles (MSNs) (Dreaden et al., 2012).

Recently, investigations combining stem cells with nanomaterials have been successfully applied in cell transplantation and regenerative medicine (Chaudhury et al., 2014). Thereinto, the MSNs feature in ordered and continuously adjustable mesoporous structure, large specific surface area, superior biocompatibility and facilitation in surface functional groups modifications, thus receive more attentions from biomedicine especially in tissue regeneration (Wang et al., 2015). Moreover, MSNs, as a FDA-approved biosafe material, has been widely used in in vivo studies with very limited toxicity compared to other nanomaterials (Liu et al., 2015). Simultaneously, subjected to the diverse functional modifications such as targeting label and fluorescence tag, MSNs has been demonstrated to effectively integrate targeting, imaging, diagnosis and therapeutics (Mamaeva et al., 2013).

Cardiovascular disease is one of the most severe threats to human health with the highest mortality (Mozaffarian et al., 2015). In according to Cardiovascular Disease Report in China 2013, patients suffering this disease were estimated to be as much as 230 million, among which myocardial...
Materials and Methods

MSNs fabrication

The method to prepare MSNs was described in our previous study (Ren et al., 2015). Briefly, 12 μl aminopropytriethoxysilane (APTES, Sigma, St. Louis, MO) was added to tetramethylrhodamine solution (TRITC, 5.5 mg in 3 ml absolute ethanol) with continuous stirring under inert atmosphere for 2 h. 2.5 ml tetraethylorthosilicate (TEOS, Sigma) was subsequently added and stirred for 12 h in the dark. Cetyltrimethyl-ammonium bromide (CTAB, Sigma) solution was prepared simultaneously by dissolving 0.5 g CTAB in 240 ml ultrapure water containing 1.75 ml NaOH (2 M, Sigma), which was subjected to rigorous stirring at 50°C. The mixture of APTES-TRITC-TEOS was then added into CTAB solution for 24 h of stirring at 50°C in the dark. The exponentially growing cells were then added into each well and incubated at 37°C for 4 h. The resultant formazan was then dissolved by addition with 100 μL of the SDS-HCl solution and allowed for incubation in the humidified chamber at 37°C for another 4 h. The absorbance at 570 nm was measured using the microplate reader (Tecan Infinite M1000 Pro, Männedorf, Switzerland).

hESCs culturing

hESCs BG01V were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in 1:1 mixture of Dulbecco’s Modified Eagle’s Medium (DMEM, Life Technology, Pleasanton, CA) and Ham’s F-12 medium supplemented with 1.2 g/l sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES, 0.5 mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.1 Mm 2-mercaptoethanol, 4 ng/ml bFGF and 15% fetal bovine serum. The cells were maintained at 37°C in a humidified incubator with 5% CO₂ supply.

 Detection of intracellular reactive oxygen species (ROS)

The exponentially growing cells were seeded into 6-well plate and subjected to the indicated treatments for 24 h. The intracellular ROS was quantified by employing ROS-sensitive dye DCFH-DA (Sigma). The fluorescent images were acquired with laser confocal microscope (Zeiss LSM 780, Germany) and the signal intensity was calculated.

Apoptosis analysis

The cell apoptosis was determined using the Annexin V Apoptosis Detection Kit (Affymetrix, Santa Clara, CA, USA) following the manufacturer’s recommendation. Briefly, the cells at log phase were harvested and washed with PBS, and then resuspended in binding buffer to prepare single-cell solution with approximate concentration of 1×10⁶ cells/mL. 5 μL of fluorescein-conjugated Annexin V was added into 100 μL of the cell suspension and incubated for 10 min at room temperature in the dark. After washing with binding buffer, cells were stained with 5 μL of propidium iodide (PI) for 10 min on ice. The apoptotic cells were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Infarction and heart failure account for 7 million and continuously increase by 10.42% annually. Cardiomyocyte death is irreversible and causal factor in heart failure after myocardial infarction, which possesses very limited regenerative capacity in terminal differentiated organs (Gatzoulis et al., 2014). Until now, the only therapeutic option for chronic heart failure is transplantation, which is dismally limited by the shortage in donor organs and expensive medical expenditures (Toyoda et al., 2013). Therefore, developments in new therapeutics to treat myocardial infarction and secondary heart failure and reduce disease-related death are still in urgent need. Recently, cardiac regenerative medicine has achieved remarkable progressions with deep insight into the biology of stem cells (Garbern and Lee, 2015). Replenishment of dead cardiomyocytes with appropriately proliferated stem cells increasingly draws attention for therapeutic purpose. Over the past decade, a variety of stem cells such as mesenchymal stem cells (Toma et al., 2002), hematopoietic stem cells (Balsam et al., 2004), adult cardiac stem cells (Beltrami et al., 2003) and embryonic stem cells (Mummery et al., 2003) have been exploited for cardiovascular diseases. However, the derived stem cells are commonly susceptible to oxidative stress under certain pathological conditions, which severely impairs their stemness including the differentiation behavior (Denu and Hematti, 2016). In view of the potential anti-oxidative activity of MSNs recently proposed by Morry et al. (Morry et al., 2015), hereby we hypothesized that MSNs could confer protection to human embryonic stem cells (hESCs) against oxidative stress, and sought to elucidate the underlying molecular mechanisms.
Effect of MSNs on hESCs against Oxidative Stress

Quantification of the intracellular glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA) level

The intracellular GSH was determined using the GSH and GSSG Assay Kit (Beyotime S0053, China), the SOD was measured with the Total Superoxide Dismutase Assay Kit with NBT (Beyotime S0109, China) and MDA was quantified by the Lipid Peroxidation MDA Assay Kit (Beyotime S0131, China) following the manufacturer’s guides.

Real time polymerase chain reaction (PCR)

The total RNA was extracted using Trizol reagent (Life Technologies) in accordance with the manufacturer’s instruction. The quality and quantity of RNAs were first determined with BioAnalyzer 2100 (Agilent, Santa Clara, CA, USA). The reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher) following the manufacturer’s guide. The real-time PCR was performed with SYBR Green Master Mix (ThermoFisher) on ABI-7900HT. The relative expression was normalized to GAPDH using 2^–ΔΔCt method. The primers information are as follows:

- Nkx2.5:
  - forward: 5'-ACATTTTACCCGGGAGCCTA-3',
  - reverse: 5'-GGCTTTGTCCACGCTCCACT-3';
- Mef2c:
  - forward: 5'-ATCCCGATGCAGACGATTCAG-3',
  - reverse: 5'-AACAGCAACAAACTTTGCT-3';
- Tbx5:
  - forward: 5'-ACTGGCCTTAATCCCAAAACG-3',
  - reverse: 5'-AACAGGAACATTGTTATCAGG-3';
- dHand:
  - forward: 5'-GAGAACCCCTACTTCCACGG-3',
  - reverse: 5'-AGGCGTAGTCGTATGGGTG-3';
- a-MHC:
  - forward: 5'-CCAGCTAAAGGCTGAGAGG-3',
  - reverse: 5'-AGGCGTAGTCGTATGGGTG-3'

Western blot

Cell lysate was prepared in RIPA lysis buffer. The protein concentration was determined using the bicinchoninic acid (BCA) Protein Assay Kit (ThermoFisher). Equal amount of protein samples were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane on ice. After brief blocking with 5% skim milk in tris-buffered saline and Polyborate 20 (TBST) buffer at room temperature for 1 h, the PVDF membrane was hybridized with indicated primary antibodies (Connexin 43, 1:1,000; GAPDH, 1:1,000; Troponin c1, 1:1,000; Cell Signaling Technology, 1:5,000) at room temperature for 1 h. After second round of washing with TBST, the target bands were visualized using the Enhanced Chemiluminescence Kit (ECL, Millipore, Billerica, MA, USA) in accordance with the manufacturer’s instruction. The relative intensity was determined by densitometry and normalized to GAPDH.

Beating percentage and beating area

The hESCs were stimulated for differentiation using hanging drop method. Briefly, hESCs were digested into single cells and hanging drops of 800 cells resuspended in 20 mL of cultivation medium without LIF were prepared. The generated embryonic bodies (EBs) were transferred to petri dishes and resuspended for 2 days. Subsequently, the EBs were plated on gelatin-coated plates and the beating behavior of EBs were observed on day 5.

Statistics

All data in this study were acquired from at least three independent experiments. The results were presented as mean±standard deviation (SD). The statistical analysis was performed with SPSS 23.0 software. One-way analysis of variance (ANOVA) followed by Turkey’s multiple comparisons test was employed for statistical comparison. The statistical significances were calculated as P values, and P<0.05 was considered statistically different.

Results

Effects of MSNs on H₂O₂-induced cell death and apoptosis in hESCs

We first evaluated the potential cytotoxicity of MSNs on hESCs in vitro by treating cells with up to 1,000 μg/mL MSNs. The cell viability was determined by MTT assay. As shown in Fig. 1a, we have observed no impact on cell viability even with the highest concentration of MSNs. Meanwhile, there was no obvious increase in cell viability upon co-incubating with MSNs, which indicated administration with MSNs alone had no impact on cell growth of hESCs. While challenged with H₂O₂, the significant inhibition of cell viability was induced in a dose-dependent manner up to 2 mM (Fig. 1b), with 0.5 mM of H₂O₂ treatment elicited about 50% decrease in term of cell viability. Thereafter, the hESCs were treated with 0.5 mM H₂O₂ for 24 h in the presence of different doses of MSNs. The cell viability was markedly suppressed upon H₂O₂ treatment, which was partially reversed by supplementation with MSNs in a dose-dependent manner (Fig. 1c). The oxidative stress has been demonstrated to stimulate cell apoptosis in addition to cell growth inhibition. Therefore, we further clarified whether MSNs conferred protective effect against H₂O₂-induced cell death. As shown in Fig. 1d with representative flow cytometric results, 0.5 mM H₂O₂ evoked notable shift
of cell population into Annexin V+/PI- quadrant, which indicated remarkable cell apoptotic signal. Consistent with antagonistic effect with H$_2$O$_2$-induced inhibition of cell viability, here we demonstrated that MSNs treatment significantly protected hESCs from apoptosis (Fig. 1e). Taken together, our results demonstrated that MSNs conferred notable stimulatory effect on cell viability and ameliorated cell apoptosis without apparent cytotoxicity.

**MSNs treatment attenuates H$_2$O$_2$-induced oxidative stress in hESCs**

Our previous data suggested the protective effect of MSNs against H$_2$O$_2$-induced cell viability inhibition and cell apoptosis. Next, we sought to investigate whether MSNs functioned via modulation of intracellular redox, which was radically changed by H$_2$O$_2$. We systematically interrogated the redox status by measurement of intracellular GSH, SOD, MDA and direct detection of ROS. As shown in Fig. 2a, the reducing equivalent GSH was remarkably depleted upon H$_2$O$_2$ treatment for 24 h, most of which was retained in the presence of 1,000 μg/mL MSNs in the culture medium. Similarly, the SOD enzymatic activity was markedly inhibited in response to H$_2$O$_2$ treatment, and reversed by MSNs (Fig. 2b). The lipid oxidation product, MDA, was significantly increased (around 1.7-fold) in H$_2$O$_2$-treated hESCs, which indicated extreme intracellular oxidative stress, and dramatically declined in the presence of MSNs (Fig. 2c). Furthermore, we labeled the ROS species with fluorescent probe to directly observe intracellular redox status, with the relative signal intensity calculated and presented in Fig. 2d. The ROS were dramatically instigated by H$_2$O$_2$ treatment in line with previous reports, which was greatly suppressed and scavenged by MSNs. To exclude the intrinsic anti-oxidative activity of MSNs under physiological conditions, we challenged hESCs with the same concentration of MSNs alone, and our results showed no influence on intracellular GSH, SOD, MDA and ROS. Therefore, MSNs antagonized H$_2$O$_2$-evoked oxidative stress and eliminated intracellular ROS production, which consequently conferred protection against oxidative damages.

**MSNs improve H$_2$O$_2$-inhibited hESCs-derived EBs-cardiomyocytes differentiation**

Next, we further evaluated MSNs influence on hESCs-derived EBs differentiation towards cardiomyocytes in the presence of H$_2$O$_2$ challenge. Quantitative real-time PCR analysis was performed to determine the relative expression levels of selected cardiac marker genes. The hESCs-derived EBs were treated with 0.5 mM H$_2$O$_2$ for 24 h in the absence or presence of 500 μg/mL MSNs. The cardiac transcription factors including Nkx2.5, Mef2, Tbx5 and dHand, all of which were well-known early cardiac progenitor cell markers, as well as the cardiomyocyte-specific structural gene α-MHC, were significantly suppressed in response to H$_2$O$_2$ treatment, which suggested impaired differentiation process of hESCs-derived EBs towards functional cardiomyocytes.
Effect of MSNs on hESCs against Oxidative Stress

**Fig. 2.** MSNs treatment attenuates H$_2$O$_2$-induced oxidative stress in hESCs. Oxidative stress was analyzed by determining GSH levels (a), SOD activity (b), MDA levels (c) and the intracellular ROS levels (d) (0.5 mM H$_2$O$_2$ for 24 h; 500 μg/mL MSNs for 5 days). Data were shown as mean±S.D. *p<0.05, **p<0.01 in comparison with control (first column). *p<0.05, **p<0.01 in comparison with H$_2$O$_2$ group (third column).

**Fig. 3.** MSNs treatment reverses the H$_2$O$_2$-induced inhibition of expressions of cardiac marker genes in EBs derived from hESCs. mRNA expressions of Nkx2.5, Mef2c, Tbx5, dHand and α-MHC were analyzed by real time PCR. GAPDH served as an internal control. Data were shown as mean±S.D. *p<0.05, **p<0.01 in comparison with control. *p<0.05, **p<0.01 in comparison with H$_2$O$_2$ group.

(Fig. 3). However, the expression of all the aforementioned genes were restored to comparable levels as control in the presence of MSNs, which unambiguously indicated an improvement with respect to cardiomyocyte differentiation imposed by MSNs co-treatment.

**MSNs improve the H$_2$O$_2$-inhibited maturation of hESCs-derived cardiomyocytes**

Our previous data supported that the presence of MSNs improved the differentiation of hESCs-derived EBs towards cardiomyocytes, which was presented by the up-regulation of early cardiomyocyte progenitor cell markers and cardiomyocyte-specific skeleton gene α-MHC. Along this direction, we sought to further clarify whether MSNs were involved in the maturation of hESCs-derived cardiomyocytes into functional cells. To this purpose, we determined the protein levels of Connexin 43 and Troponin C1 by immunoblotting, which were physiological important in the structural organization of cardiomyocytes. As shown in
Fig. 4a and b, H$_2$O$_2$ treatment dramatically inhibited both Connexin 43 and Troponin C1 expression, which was in agreement with previous observation that H$_2$O$_2$ impeded hESCs-derived EBs differentiation. In the presence of MSNs, the expression of both proteins was significantly stimulated, which suggested that MSNs promoted the maturation of hESCs-derived cardiomyocyte in addition to differentiation. Likewise, we ruled out the potential effect of MSNs alone on cardiac structural organization by directly applying the same concentration of MSNs on the derived cardiomyocytes. Here we demonstrated that MSNs improved the maturation of cardiomyocytes in vitro as well.

**MSNs treatment enhances the H$_2$O$_2$-inhibited beating properties of EBs**

Our previous data suggested that MSNs improved hESCs-derived EBs differentiation and maturation of cardiomyocytes, which was greatly compromised by H$_2$O$_2$ treatment in our system. Next, we sought to determine the effect of MSNs on functioning of hESCs-derived cardiomyocytes in terms of beating properties. Both the beating number and beating area indices were visually measured on day 10 of differentiation in the presence and absence of MSNs. As shown in Fig. 5a and b, the group treated with 0.5 mM H$_2$O$_2$ showed significant decrease in the percentage of beating cell clusters and beating area compared to the control. The compromised beating characteristics was almost completely restored in the presence of 500 μg/mL MSNs, while individual application of MSNs did not alter the beating behavior of hESCs-derived EBs. Therefore, our data clearly demonstrated that MSNs treatment enhanced the inhibition of beating properties of hESCs-derived EBs upon H$_2$O$_2$ treatment.
Discussion

Cardiovascular diseases have imposed heavy burden on individual health and socio-economy (Kathiresan and Srivastava, 2012). Despite of tremendous improvements in the clinical management of this disease, therapeutic options for the patients with severe myocardial infarction extremely rely on donor organ transplantation with very limited sources and expensive medical expenditures. Therefore, alternative remedies including tissue engineering and regenerative medicines are practically imperative. Over the past decade, numerous efforts have been invested into exploring a variety of human stem cells for therapeutic application for this disease, while some manifested promising results and some with disappointing outcomes. The hESCs increasingly attracted great attention for this purpose predominately due to its totipotency to differentiate into all three primary germ layers and the potential to generate all cell types in vitro (Chong et al., 2014). However, hESCs are susceptible to oxidative damage which radically impairs its stemness especially differentiation behavior, which highlights the necessity of maintaining the environmental redox homeostasis during in vitro cultivation and transplantation of hESCs.

MSNs has been extensively investigated as drug nanocarrier due to its unique and tunable mesoporous structure, high loading capability and biocompatibility for wide range of human diseases including diabetes, cancer and tissue injury. Our previous study demonstrated that ascorbic acid delivered by mesoporous silica nanoparticles induced differentiation of human embryonic stem cells into cardiomyocytes (Ren et al., 2015). Beck et al. reported that serum protein adsorption enhanced active leukemia stem cell targeting of MSNs (Beck et al., 2017). Durfee et al. showed that MSNs-supported lipid bilayers (protocells) were capable of active targeting and delivery to individual leukemia cells (Durfee et al., 2016). Chen et al. employed functionalized MSNs to enhance sensitivity of cancer stem cells to chemotherapy (Chen et al., 2016). Datz et al. demonstrated that genetically designed biomolecular capping system for MSNs enabled receptor-mediated cell uptake and controlled drug release (Datz et al., 2016). And Cheng et al. reported that 5-Azacytidine delivered by MSNs regulated the differentiation of P19 cells into cardiomyocytes (Cheng et al., 2016). With regard to stem cells, Chen et al. demonstrated a new method for non-viral cell labeling and differentiation agent for induced pluripotent stem cells based on MSNs (Chen et al., 2013a), and Kim et al. evaluated the efficacy of MSNs in delivering BMP-2 plasmid for in vitro osteogenic stimulation of mesenchymal stem cells (Kim et al., 2013). In addition to its critical role as drug carrier, here we evaluated the potential influences of MSNs on hESCs. Our results demonstrated no apparent cytotoxicity associated with MSNs up to 1,000 μg/mL, which was consistent with its well-known biosafety in our setting.

Intriguingly, Morry et al. recently demonstrated that the MSNs core imparted an anti-oxidative property by scavenging ROS and subsequently reducing NOX4 levels in the in vitro fibrogenesis model, which indicated the potential applications of MSNs alone in modulating local environmental redox homeostasis (Morry et al., 2015). Along this direction, here we challenged hESCs with H₂O₂ in vitro, which stimulated intensive intracellular oxidative stress and in turn inhibited cell viability and induced cell apoptosis. The intracellular reducing equivalent of GSH and enzymatic activity of SOD were significantly suppressed, while the lipid oxidation product MDA and ROS were stimulated in response to H₂O₂ treatment. The differentiation and maturation of cardiomyocytes were determined by respective marker genes, which were remarkably inhibited by H₂O₂. Accordingly, the beating behavior was markedly compromised as well. All the aforementioned phenotypes were greatly reversed in the presence of MSNs in the culture medium, which suggested the protective effect of MSNs on hESCs against oxidative stress at least in vitro and held great promise for future exploitation in hESCs transplantation.

Accumulating evidences have displayed the intrinsic anti-oxidative properties of inorganic nanoparticles including nickel (Saikia et al., 2010), platinum (Nomura et al., 2011), ceria (Heckert et al., 2008), yttria (Schubert et al., 2006) and mesoporous silica (Sadeghnia et al., 2015). The MSNs are the most appropriate for biomedical applications in view of its biosafety, biocompatibility and biodegradation. Intensive efforts have been focused on removing intracellular ROS using the modified MSNs. For instance, Chen et al. developed a new strategy by embedding superoxide dismutase in MSNs to efficiently deliver anti-oxidative enzyme into cells and confer protection from ROS (Chen et al., 2013b). Shen et al. demonstrated horseradish peroxidase-immobilized magnetic MSNs as a potential candidate to eliminate intracellular ROS (Shen et al., 2015). And Ebabe et al. reported that MSNs functionalized with anti-oxidants, such as caffeic acid and rutin, could efficiently generate lower oxidative stress impact on cells (Ebabe Elle et al., 2016). Noteworthily, here we demonstrated that H₂O₂ treatment significantly attenuated SOD expression, which was conversely induced in the presence of MSNs. Our results suggested that MSNs might confer protection against oxidative stress via modulation of SOD activity, which was in contrast to the previous study in melanoma, wherein MSNs treatment inhibited NOX4 mRNA expression, the key enzyme that catalyzed the reduction of oxygen into endogenous ROS and implicated in the pathogenesis of various human diseases (Huang et al., 2010).

In conclusion, in this study we demonstrated the protective effect of MSNs on hESCs against oxidative stress and suggested the potential therapeutic value of MSNs for further investigations.

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**Disclosure of potential conflicts of interest**

The authors declare that they have no conflict of interest.

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