

Original Article

Evaluation of the Capability of the Wharton's Jelly Mesenchymal Stem Cell Aggregates to Express the Markers of Three Germ Cell Lineages

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Background: The ability of stem cells to differentiate into different cell types makes them a key component of healing damage in regenerative medicine. As human umbilical cord Wharton's jelly (HUCWJ) is available non-invasively, HUCWJ does not raise any ethical issues with higher differentiation potential compared to adult stem cells. With the ability to express embryonic stem cell markers, HUCWJ can be considered as a good candidate in regenerative medicine applications. The objective of this study was to find if these cells form cell aggregates with the same features as that formed by embryonic stem cells (embryoid body) and could form three germ layers.

Methods: Eighteen umbilical cords were of healthy infants with parent permission. The umbilical cords were cut into small pieces and the explants were cultured. At the third passage, 1000, 5000 and 10000 cells/ 20 μ L were cultured in hanging drops for 3 days. Then, they were incubated for additional 3 days in non-adhesive dishes. As the center of cell aggregates formed from 5000 and 10000 cells were darker than those formed from 1000 cells, this study focused on the aggregates formed by 1000 cells for further assessments. The immunocytochemistry and flowcytometry were performed using 3 color antibodies to detect the markers for three germ cell lineages.

Results: The immunohistochemistry data showed that the embryoid-body-like aggregates expressed a low amount of ectodermal and endodermal markers and most of the cells expressed mesodermal markers. The flowcytometry percentage of the cells in each aggregate that expressed ectodermal marker Otx2 was 17.1% and endodermal marker, Sox 17 was 5.49%. The frequency of cells expressing mesodermal marker Brachyury was high (75.0%). Flowcytometry also showed the percentages by mathematical evaluation and we did this three times for our result accuracy.

Conclusion: These aggregates mainly kept their mesenchymal state and showed a poor differentiation potential toward ectoderm and endoderm identity.

Keywords: Brachyury, Cell aggregates, Embryoid-body, GATA4, HAND1, Wharton's jelly

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Introduction

Nowadays, stem cells have been widely used in many regenerative medicine approaches. Stem cells have various sources including inner cell mass of the blastocyst, fetal organs, medical waste tissue after delivery and adult organs. Human umbilical cord Wharton's jelly (HUCWJ) is one of the medical waste tissues that can be considered as an appropriate source of stem cells without ethical issues. Stem cells derived from HUCWJ are heterogeneous and multipotent cell populations with the ability to differentiate into derivatives of three embryonic germ layers.¹ The stem cells derived from HUCWJ can express both mesenchymal stem cell (MSC) and embryonic stem cell (ESC) markers especially those involved in pluripotency,² as well as hematopoietic markers.³ Although, most of the cells in Wharton's jelly showed MSC behavior and

expressed MSC markers, but the origin of the HUCWJ including those expressing ESC marker was unclear, but it has been suggested that they may originate from migrating primordial germ cells or hematopoietic stem cells derived from yolk sac and cross through umbilical cord toward their destinations in gonadal ridge or mesonephros-aortogonad organ, respectively. Both primordial germ cell and ESCs have the same properties.⁴

The culturing system has been shown to influence the physiology and phenotype of stem cells derived from HUCWJ in many ways. For instance, post confluence culturing of HUCWJ has been shown to produce structures similar to embryoid body that are formed by ESCs. The MSCs derived from other sources such as bone marrow derived MSCs cannot form embryoid-like structures. These embryoid-like structures could express

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ectodermal markers.⁵ Culturing MSCs derived from HUCWJ in aggregates has been shown to modify the gene expression pattern,³ and as a result it may influence their differentiation capabilities,^{6,7} and functions.⁸ Besides, HUCWJ has been also shown to express different integrins when cultured in cell aggregates versus 2D conventional culture condition. Integrins have impact on cell shape,^{9,10} as well as pluripotency.¹¹ Therefore, the culture system can influence many behavioral aspects of stem cells derived from HUCWJ.

Differentiation of ESCs can occur after formation of 3D aggregates named embryoid bodies (EB).¹² EB is an *in vitro* biomimicry of pre-implantation embryo which is used for differentiating of pluripotent stem cells.^{13,14} Within the EB microenvironment, a wide range of cell types from all cell lineages differentiate.¹⁵ As the stem cells derived from HUCWJ have some similarities with ESCs, it was hypothesized that the cells behave similarly to ESCs and at least those expressing ESC markers could differentiate into ectoderm and endoderm rather than mesoderm and form the layers similar to those in EB. This may change the differentiation potential of HUCWJ-MSCs. To check this hypothesis, we designated a study to find if HUCWJ-MSC aggregates could form three embryonic germ layers like EB.

Materials and Methods

Isolation and HUCWJ-MSC Culture

Human umbilical cords were achieved from 18 full-term infants born via cesarean sections at the University Hospital with inform consents from their parents according to the ethic committee guidelines. Wharton's jelly is a kind of fetal connective tissue rich in MSCs which were used in regenerative medicine recently. The umbilical cord is a waist product that can be used in MSC differentiation and cell therapy. The healthy infants born without any problems with parental permission were selected for umbilical cord using in our research.

The cords were washed twice in PBS solution and cut into 2 cm pieces. The vessels of each pieces were removed, the Wharton's jelly was cut into 4–5 mm segments and each one was explanted in the flask. After 5–10 minutes, 5 mL α -MEM medium (Shell max) were added to each flask. The medium was supplemented with 10% fetal bovine serum (FBS, Gibco), 1% L-glutamine, 100 g/mL antibiotic-antimycotic solution. The T25 flasks were incubated at 37°C and 5% CO₂ for 7–10 days and the medium was changed every 3 days. The cells started growing from the edge of each explant. The cells were sub cultured along with dissociation with trypsin-EDTA when they reached confluence up to 80%-90%.

MSC Characterization

At passage 3, the cells were suspended in the phosphate buffer saline (PBS) containing 2% FBS. The cell

suspension at the density of 1×10^6 cells/mL was washed in blocking solution; cold PBS containing 10% FBS, for 20 minutes. Then, the cell number was adjusted at 5×10^5 cells per each flow cytometry tube. The cells were labeled with ready to use FITC (fluorescein isothiocyanate)-conjugated anti-CD44, anti-CD90, and anti-CD144, PE (phycoerythrin)-conjugated anti-CD34, anti-CD73, and anti-CD106, and PerCP (Peridinin Chlorophyll Protein complex)-conjugated anti-CD105 antibodies (all from Abcam, UK, Cambridge). The isotype antibodies were also used to omit background. The samples were incubated for 25 min in the dark at room temperature. Then, the cells were washed with PBS, centrifuged at 300 g for 10 minutes, fixed with 1% paraformaldehyde for 15 minutes and finally washed with cold PBS. Then, the samples were resuspended in PBS containing 10% FBS, and analyzed by a flow cytometer (FACS Calibur™, BD Biosciences). The data were then analyzed by FlowJo software (TreeStar, Ashland, OR, USA).

Adipogenic and Osteogenic Potential HUCWJ-MSCs

To assess multipotency capability of the WJ-MSCs, their ability to differentiate into osteoblasts and adipocytes were also evaluated. After harvesting and counting the cells, they were seeded at the density of 3000 cells/well of a 24 well plate, then, the adipogenic (StemCell Technologies, Inc., Canada) differentiation medium were added and the cells were incubated in 37°C and 5% CO₂ for 4 weeks. Then, the differentiated cells were washed with PBS and fixed with 4% paraformaldehyde for 1 h at 4°C. The cells were then incubated in oil red (Sigma, USA) for 15 minutes. The cells also were checked for differentiation capability to osteoblasts. To do this, they were seeded at a density of 3000 cells/well of a 24 well plate and, then exposed to the osteogenic (MACS, Germany) differentiation medium for 4 weeks in 37°C and 5% CO₂. Then, they were fixed with 96% methanol for 10 minutes. After washing with PBS, the cells were incubated with 0.5% Alizarin red (Sigma, USA) for 24 hours in 4°C. The cells cultured in osteogenic medium differentiated into osteoblast and restored Ca²⁺.

Cell Viability

We used Trypan blue to evaluate cell viability. The dye exclusion test is used to determine the number of viable cells, which live cells possess intact cell membranes that exclude certain dyes, whereas dead cells do not. The live and dead cells, were counted using a haemocytometer and a light microscope after mixing with Trypan blue and the percentage of viability was calculated and then aliquoted into 1000, 5000 and 10000 cells for each aggregates formation.

Primary Cell Aggregates Formation

At the third passage, an aliquot of 1000, 5000 and 10000 cells were used to form aggregates. First, the cells were

trypsinized, centrifuged at 300 g and counted to adjust the cell number per 20 μ L. The hanging drop (HD) method was used to form cell aggregates. The cell suspension was dripped on the lid of a 100 mm petri dishes and the half of dishes was filled with PBS and incubated at 37°C and 5% CO₂ for 3 days. Thereafter, the primary aggregates were collected and transferred to non-adhesive plates (Griner, Germany); 4 mL medium was added to culture dishes and incubated for additional 3 days.¹⁶

Morphological Study

The cell aggregates were collected and the morphology of the aggregates was studied using a stereomicroscope. The aggregates were fixed with 4% paraformaldehyde and paraffin blocks were prepared. Then, they were cut with microtome at the thickness of 5 μ m. The H&E staining was done and the morphology of aggregates was studied with light microscope.

Immunocytochemistry

For immunostaining, the human 3 germ layer 3-color immunocytochemistry antibodies were used (R&D systems). The kit contains 6 fluorochrome-conjugated antibodies that conjugated with FITC/PE and can detect ectoderm, mesoderm and endoderm markers. FITC/PE-conjugated anti-human SOX1 and Otx2 antibodies for ectoderm; mesoderm: FITC/PE-conjugated anti-human HAND1 and Brachyury antibodies for mesoderm and FITC/PE-conjugated GATA4 and Sox17 for endoderm.

The cell aggregates was fixed in PBS containing 4% paraformaldehyde for 20 min. Then, the non-specific binding sites were blocked with PBS containing 10% horse serum, 0.3% Triton X-100, and 1% BSA for 45 minutes. A series of cell aggregates were incubated with each corresponding conjugated antibodies at 1:10 dilution in blocking buffer for 3 hours at room temperature and dark. After washing with PBS containing BSA, the cells were counterstained with Hoechst 33343 (Sigma) for 5 minutes and were observed by a fluorescent microscope (Zeiss, Germany), using the appropriate excitation wavelength filters.

Flowcytometry

The cell aggregates were incubated with EDTA/Trypsin for 5 min. Pipetting was done to disaggregate of cells and the single cell suspension was prepared. They were then permeabilized with PBS containing 0.5% Triton and centrifuged at 1200 rpm. The cells were treated with the same procedure performed for detection of MSC CD markers. The anti-Brachyury, Oct4 and Sox17 antibodies were used for flow cytometry.

Results

Culture and Characterization of HWJMSCs

The first cell buds were observed around each explant

after 7–10 days. HUCWJ-MSCs had fibroblastic-like phenotype (Figure 1) and at the first passage, the cells were small and fusiform. After third passage, the cells seem fully expanded with many cytoplasmic processes.

The flow cytometry of the HUCWJ-MSCs revealed no reactions for hematopoietic lineage (CD34) or endothelial cell (CD144) markers, but they showed a positive reaction for CD105, CD106, CD44, CD73 and CD90 (Figure 2).

Osteogenic and Adipogenic Differentiation Results

The differentiation capacities of HUCWJ-MSCs toward adipogenic and osteogenic lineages were evaluated by oil red staining and Alizarin Red/S, respectively (Figure 3). The cells also showed that they could differentiate and store lipid droplets after the exposure with adipogenic medium. The osteogenic differentiation potential also confirmed the alizarin red S stained the Ca²⁺ deposit. These data confirmed the pluripotency of the isolated cells.

Cell Aggregate Assessments

Incubating the cells in hanging drop showed that they could form cell aggregates. They were assembled as large 3D spherules in each drop. The center of each aggregate showed a darker area which may indicate cell degeneration. Comparison of the cell aggregates formed from different cell numbers showed that the aggregates contained 1000 cells formed earlier than those contained 5000 and 10 000 cells. The center of aggregates that contain 5000 and 10 000 cells was darker than the center of 1000-cell aggregates. Also, the cell aggregates which contained 1000 cells were more uniform than the 5000 and 10 000-cell aggregates (Figure 4). After incubating the cell aggregates in non-adhesive plates, the same results were also observed. Therefore, the aggregates contained ,000 cells were used for evaluation of expression of 3 germ layer markers.

The H&E staining revealed the presence of the cells at the center of the aggregates formed from ,000 cells. The cells also formed an epithelioid structure at the periphery of each aggregate (Figure 5). Some small spaces started to form within the central part of the aggregates.

Immunocytochemistry and Flowcytometry

The aggregates formed by 1000 cells expressed both ectodermal markers; Sox1 and Otx2, with “strong” and

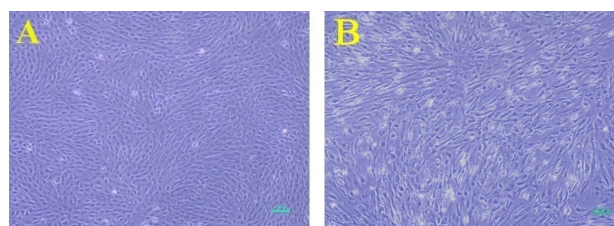


Figure 1. The Morphology of Wharton's Jelly-Derived Mesenchymal Stem Cells at (A) Primary Culture and (B) 3rd Passage.

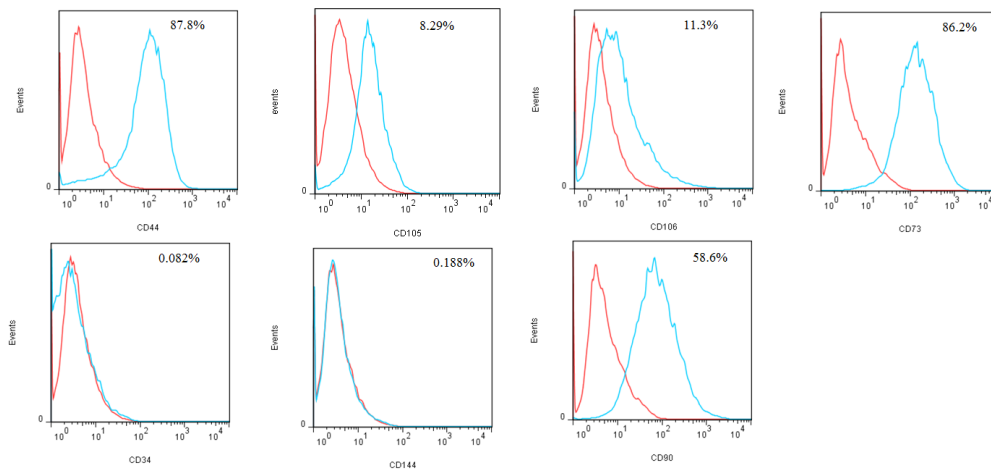


Figure 2. The Frequency of the Positive Cells for CD Markers by Flow Cytometry. The HUCWJ-MSCs expressed CD44, CD105, CD106, CD90, and CD73 but they expressed CD34 and CD144 with less frequency.

“weak” intensities, respectively. Also, these cell aggregates expressed both mesoderm markers; Brachyury and HAND1 with “strong” and “weak” intensities, respectively. The cell aggregates stained weakly with both endodermal markers, GATA4 and Sox17 (Figures 6 and 7).

The flowcytometry also confirmed the results of immunocytochemistry. The percentage of the cells in each aggregate that expressed ectodermal marker, Otx2, was 17.1% and endodermal marker, Sox 17, was 5.49%, which both of them were low. The frequency of the cells expressed mesodermal marker, Brachyury, was high (75.0%). Therefore, most of the cells kept their mesodermal properties in the cell aggregates (Figure 8).

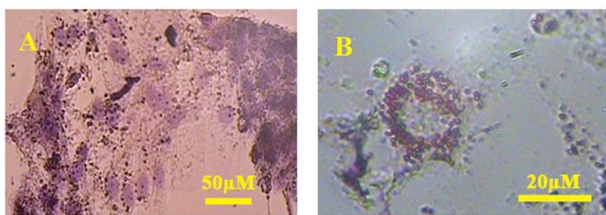


Figure 3. Osteogenic differentiation was confirmed by staining of Calcium deposits with Alizarin Red/S (A). Oil Red O showed the presence of lipid droplets in the cells cultured in adipogenic medium (B).

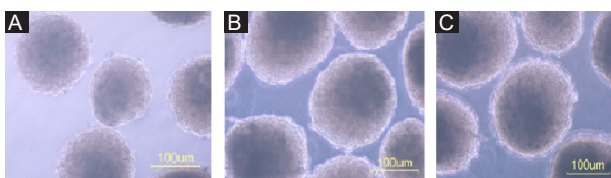


Figure 4. Comparing the Aggregates Formed from 1000, 5000 and 10000 Cells. The cell aggregates formed from 1000 cells (A) were more uniform and rounder than the other two cell aggregates. The center of the aggregates formed by 5000 (B) and 10000 (C) cells was darker than that in the aggregates formed by 1000 cells.

Discussion

HUCWJ is one of the medical waste tissues that can be considered as an appropriate source of stem cells in cell therapy and regenerative medicine without ethical concerns. HUCWJ-MSCs have been shown to express surface CD markers that are common in the MSCs derived from the other sources and umbilical cord has been considered as a source for MSCs.^{4,17}

Data from the current study showed that most of the cells in cell aggregates expressed mesenchymal cell markers although a low number of cells also expressed ectodermal and endodermal cell markers. However, the umbilical cord is a route for primordial germ cell and hematopoietic stem cell migration from where they originate.⁶ According to this finding, it may be suggested that some primordial germ cell and hematopoietic stem cells remain in the cord blood and form a small population the stem cells.⁴ Therefore, we hypothesized that at least a subpopulation of these cells may behave as primordial germ cells or ESCs when they are cultured in hanging drops. It can be hypothesized that HUCWJ-MSC aggregates act as EB formed from ESCs, so that they could differentiate into three germ layers when they are cultured in hanging drops.

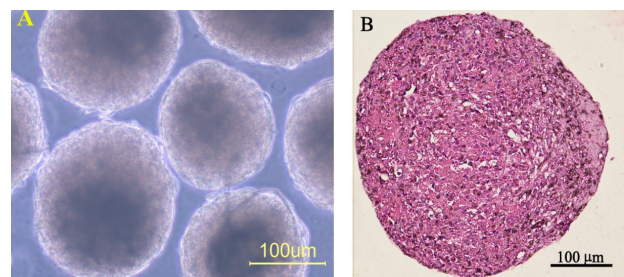


Figure 5. The Histological Morphology of Aggregates Formed from 1000 cells. The fresh aggregates, A) fixed aggregates, B) H&E staining.

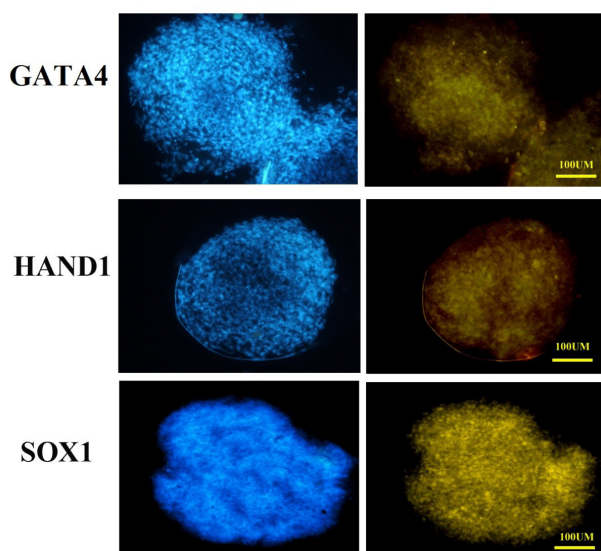


Figure 6. Immunostaining of Aggregates Formed by 1000 Cells. The cell aggregates had a weak expression of GATA4 and HAND1; however, the expression of Sox1 was high.

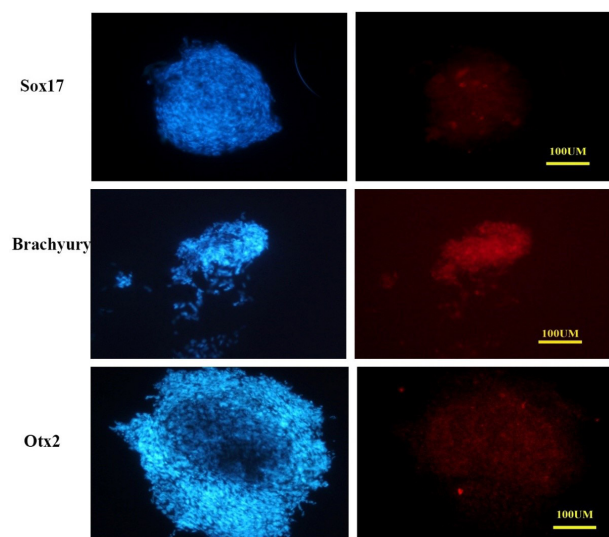


Figure 7. Immunostaining of Aggregates Formed by 1000 Cells. The cell aggregates expressed a high level of Brachyury T but they stained weakly by anti- Sox17 and Otx2 antibodies.

Our data showed that HUCWJ-MSCs could form cell aggregates. The spontaneous formation of EB-like aggregates also has previously been shown by Adamzyk et al when they cultured in post-confluent conditions for 6 weeks. They showed that both spontaneously formed cell aggregates and subconfluent HUCWJ-MSCs expressed ectodermal markers.⁵ Our previous study also showed that naïve HUCWJ-MSCs also expressed some endodermal markers in 3D culture as well.³ Therefore, the presence of low percent of cells that were positive for ectodermal and endodermal markers in cell aggregates may be due to the intrinsic features of HUCWJ-MSCs and it may not depend on the culture conditions. On the other hand,

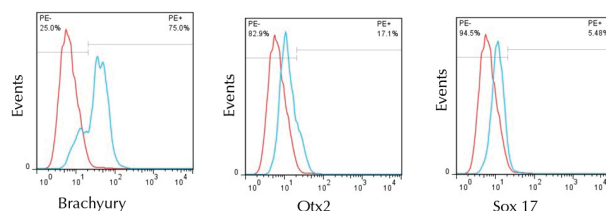


Figure 8. Flowcytometry of the aggregates formed by 1000 cells showed that the frequency of the cells expressing Brachyury was high; however, a lower percentage of the cells expressed Otx2 and Sox17.

it was shown that culturing the HUCWJ-MSCs in cell aggregates change the functional aspect of the cells such as changes in immunomodulatory properties,⁶ integrin expression pattern⁹ and hepatogenic differentiation potential.¹⁸ Therefore, cell-cell interaction in 3D culturing system may influence cell expression pattern of germ layer markers.

HUCWJ-MSC aggregates showed a dark area at the center that may indicate cell death due to nutrient diffusion limitation as it happens in EB formed from ESCs. The rate of apoptosis is related to the size of cell aggregates. In cell aggregates with 1000 cells, the increase of FBS concentration in the culture medium can accelerate the survival rate of the cells and delay apoptosis and cavity formation. Besides, the cavity size has been shown to depend on the culture condition such as the presence of specific growth factors.¹⁹ Central part of the larger aggregates has less availability to nutrients. H&E staining of sections showed an epithelioid layer at the periphery of the aggregates and some small cavities at the center. The formation of epithelioid layer can limit nutrient diffusion and lead to cavity formation at the center of each aggregate.²⁰

Both immunohistochemistry and flowcytometry revealed these cell aggregates could not form visceral endoderm and ectodermal layers like EBs and stay in form of simple cell mass, but they expressed some markers of all 3 embryonic germ layers. A large population of HUCWJ-MSCs in cell aggregates were mesenchyme rather other cell lineages.

In conclusion, these findings showed that the cell aggregates preserved their mesenchymal character and could not rearrange into structures that were like EB. Despite formation of epithelioid layer surrounding the cell aggregates, the endodermal marker expression was not limited to this epithelioid structure. However, most of the cells expressed Oct4 located at the center of aggregates.

Authors' Contribution

SB contributed to conception, design and final approval and performed the data analysis, TTKh contributed to design, data analysis and manuscript correction and FRT contributed to drafting of the manuscript, and data collecting.

Conflict of Interest Disclosures

The authors have no conflicts of interest.

Ethical Statement

The study was approved by the ethics committee of Shiraz University of Medical Sciences.

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