DOI: 10.17816/PTORS5431-37

THE USE OF CELLULAR TECHNOLOGIES IN THE TREATMENT OF CHILDREN WITH CONGENITAL CLEFT PALATE

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Received: 27.09.2017 Accepted: 16.11.2017

Background. Mesenchymal stromal cells (MSCs) are multipotent stem cells capable of differentiation in the osteogenic, chondrogenic, and adipogenic directions that are widely used in the development of new cellular biomedical technologies.
Aim. We investigated the effect of MSCs on osteogenesis in the congenital defect of the alveolar process of the upper jaw with the aim of improving the treatment results for children with congenital cleft palate.
Materials and methods. At the department of maxillofacial surgery of the Turner Institute for Children's Orthopedics, 46 patients with a diagnosis of congenital cleft palate were observed in 2017. Six patients with congenital cleft palate in the region of the defect of the hard palate and the alveolar process of the upper jaw underwent uranoplasty and implantation of a mixture (1 : 4) of MSCs and preosteocytes derived from them on the osteogenic membrane. The control group consisted of 40 age-matched patients who underwent the same surgery but without the use of MSCs. The distance between the cleaved portions of the alveolar process of the upper jaw ranged from 0.5–1.0 cm. The follow-up period was 6–9 months.
Results. On X-ray examination 6–9 months after the operation in the bone defect area and implantation of MSCs in all patients, tissue with a density corresponding to that of bone was found. In the control group, bone tissue was not formed in the diastasis of the alveolar bone. There were no significant differences in the timing of wound healing and course of the postoperative period.
Conclusion. Tissue engineering helped in the treatment of severe congenital malformations of the maxillofacial area. There are good prospects for using MSCs in the surgical treatment of defects of the facial skeleton.

Keywords: congenital cleft palate; cell technologies; mesenchymal stromal cells; tissue engineering; restorative surgery; children.

ИСПОЛЬЗОВАНИЕ КЛЕТОЧНЫХ ТЕХНОЛОГИЙ ПРИ ЛЕЧЕНИИ ДЕТЕЙ С ВРОЖДЕННЫМИ РАСЩЕЛИНАМИ НЕБА

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Статья поступила в редакцию: 27.09.2017 Статья принята к печати: 16.11.2017

Актуальность. Мезенхимные стромальные клетки (МСК) являются мультипотентными стволовыми клетками, способными к дифференцировке в остеогенном, хондрогенном и адипогенном направлениях и широко используются для разработки новых клеточных биомедицинских технологий.
Цель работы — улучшение результатов лечения детей с врожденными расщелинами неба, изучение влияния мезенхимных стволовых клеток на остеогенез в области врожденного дефекта альвеолярного отростка верхней челюсти.

■ For citation: Stepanova YuV, Tsyplakova MS, Usoltseva AS, et al. The use of cellular technologies in the treatment of children with congenital cleft palate. *Pediatric Traumatology, Orthopaedics and Reconstructive Surgery*. 2017;5(4):31-37. doi: 10.17816/PTORS5431-37

Материалы и методы. На отделении челюстно-лицевой хирургии НИДОИ им. Г.И. Турнера в 2017 г. наблюдались 46 пациентов с диагнозом «врожденная расщелина неба». При проведении операции уранопластики 6 пациентам с врожденными расщелинами неба в область дефекта твердого неба и альвеолярного отростка верхней челюсти была имплантирована смесь (1 : 4) МСК и полученных из них преостеоцитов на остеогенной мембране. Контрольная группа составила 40 человек, оперированных по аналогичной методике, но без применения МСК. Все пациенты были одной возрастной группы. Расстояния между расщепленными участками альвеолярного отростка верхней челюсти было 0,5–1,0 см. Срок наблюдения составил от 6 до 9 мес.

Результаты. При рентгенологическом обследовании через 6–9 месяцев после операции в области костного дефекта после применения МСК у всех пациентов обнаружена ткань, по плотности соответствующая костной. В контрольной группе в диастазе альвеолярного отростка костная ткань не формировалась. Достоверных различий в сроках заживления раны и течении послеоперационного периода не было.

Заключение. Тканевая инженерия помогает в лечении наиболее тяжелой врожденной патологии челюстно-лицевой области. Открываются хорошие перспективы использования МСК для оперативного лечения дефектов лицевого скелета.

Ключевые слова: врожденная расщелина неба; клеточные технологии; мезенхимальные стромальные стволовые клетки; тканевая инженерия; реконструктивно-восстановительная хирургия; дети.

Introduction

Stem cells are undifferentiated cells that can self-renew, mitose and differentiate in osteogenic, chondrogenic, and adipogenic directions. They are widely used to develop new cellular biomedical technologies.

Scientists A.A. Maksimov and A.Ya. Friedenstein discovered two types of stem cells: hematopoietic stem cells which are the precursors of blood cells, and mesenchymal (or stromal) stem cells which are long-living stem cells that rarely divide and are constantly circulating in the bloodstream.

Mesenchymal stromal cells (MSCs) are successfully used in the treatment and prevention of various diseases [1]. Use of MSCs is one of the most promising areas of modern medicine. A significant corpus of scientific evidence indicates the effectiveness of MSCs for treating a number of serious diseases, including diseases of the maxillofacial area (MFA) and the musculoskeletal system. Osteogenic differentiation of MSCs presupposes the ability of these cells to "develop" into osteoblasts. This differentiation determines the prospects for repair or regeneration of bone tissue, and for treating imperfect osteogenesis. To induce osteogenic differentiation of MSCs, dexamethasone, ascorbic acid and β-glycerophosphate are added to the culture medium. Differentiation of MSCs into osteoblasts is confirmed by the emergence of osteocalcin mRNA, cdfa1, increased alkaline phosphatase activity, and the presence of extracellular precipitates of calcium salts [2–5].

To obtain MSCs, the following donor areas are most often used: bone marrow (the main source) and adipose tissue, which is the most accessible biological material. MSCs derived from adipose tissue effectively differentiate into bone cells, and stimulate vascular growth through the secretion of the vascular endothelial growth factor (VEGF) which provides greater efficacy.

The hard and soft palates are structures that separate the oral and nasal cavities. The formation of the palate occurs during weeks 8–15 of fetal embryonic development. Severe anatomical and functional disorders occur in the formation of congenital cleft palate. Here there is communication of the oral and nasal cavities, impairing the function of the palato-pharyngeal ring. Congenital cleft palate also produces further impairment of the vital functions of respiration, deglutition, and speech. Surgical treatments for congenital cleft palate seek to separate the oral and nasal cavities.

One of the problems of patients with complete unilateral and bilateral cleft palates is the absence of bone tissue and congenital malformation of the upper jaw in the region of the alveolar process. Currently, the most popular methods of alveoloplasty use bone autografts from the iliac crest, and rib autografts. However, the results of these surgical interventions are not always satisfactory and can lead to the formation of defects in the anterior part of the palate and oronasal fistulas.

When treating pediatric patients with congenital pathology of MFA (congenital cleft lip and palate), MSCs are used in surgeries to close defects of the hard palate, as well as when closing the fissures of alveolar process of the congenitally malformed upper jaw [6]. Lack of bone is a problem that

must be overcome in order to restore the dentition or facial structures. The integrity of the alveolar process of the upper jaw is important for dentition implantation and restoration. Formed bone serves as a support for the base of the nose wing, which greatly improves the aesthetic results of treatment of pediatric patients with congenital cleft lips and palates.

Materials and methods

To study the effect of MSCs on the formation of bone tissue in pediatric patients with various forms of cleft palate, 6 patients were selected (Figures 1, 2). One patient was observed with a congenital middle cleft palate, 2 patients had congenital complete unilateral cleft palates, and 3 patients had complete congenital bilateral cleft palates. The age of the patients ranged from 2 to 4 years. All children were observed by the doctors of the clinic since birth, where they underwent comprehensive surgical and orthodontic treatments. Criteria for selection of the timing of surgical treatments were the patients' general somatic health, dentoalveolar status, and the absence of alveolar arch deformities.

All patients ($n = 6$) underwent sparing one-stage uranoplasty with mesopharyngoconstriction. We used the method of sparing uranoplasty which enables the formation of an anatomically correct fully functional palate for treating clefts of any shape (patent for invention No 2202965). The fissures of the alveolar process of the upper jaw was simultaneously closed in pediatric patients with complete unilateral and bilateral cleft palates ($n = 5$).

All patients agreed to participate in the study and provided written informed consent. We obtained permission from the local ethics committee for all study activities.

We used autologous stromal cells derived from the autologous adipose tissue of 4 patients, and allogeneic MSCs from donor umbilical cords in 2 patients. MSCs were isolated from adipose tissue and the umbilical cord.

Allogeneic MSCs of the perivascular space of the umbilical vein (Pokrovsky Bank of Stem Cells, SPb, Russia) were obtained from mothers who underwent uncomplicated labor. The cords were transported from the maternity hospitals of St. Petersburg in a sterile container with a 1% solution of penicillin, streptomycin and fungizone in physiological saline. The umbilical vein of the cord was washed with Versene solution, filled with a 0.2% solution of Type I and IV collagenases in phosphate buffered saline (PBS), terminated from both sides and incubated for 1 hour at 37°C. The resulting cell suspension was washed from the enzyme by centrifugation (400 g, 10 min) and inoculated in vials at a density of 100–400 thousand cells/cm^2. Then the vein lumen was re-filled with a solution of collagenases, terminated, and the steps described above were repeated (RF patent No 2620981). Selection of MSCs was performed on the basis of their ability to adhere and proliferate. All samples were tested for the absence of HIV 1, 2, hepatitis B, C; syphilis, and CMV, regardless of the mother's examination data. An analysis was also performed for bacterial and fungal contamination of biological material and karyotyping of the initial sample.

MSCs of the primary cultures were grown in the nutrient medium of AdvanceSTEM Mesenchymal Stem Cell media (HyClone, USA) containing 10% serum substitute AdvanceSTEM Mesenchymal Stem Cell Supplement (HyClone), 50 U/ml of penicillin and 50 μg/ml of streptomycin at 37°C in the atmosphere of 5% CO_2 and 5% (hypoxic conditions) O_2 with the use of multi-gas incubators (BBD 6220, Thermo Scientific, USA). The medium was changed after 3 days of explantation. Upon reaching 70–80% confluence of the monolayer, MSCs were reinoculated (passaged) at a density of 1000 cells/cm^2 and cultured further. In total, no more than 4 reinnoculations (passages) were allowed during cultivation. Three days before the introduction of the cells to the patient, the culture medium in the primary culture was replaced with the so-called xeno-free medium, containing no animal components (without xenogenic materials, without animal components) StemPro® MSC SFM XenoFree Kit (Life Technologies, USA). Before delivery to the patient, the material was repeatedly tested for infectious agents, karyotyped and immunophenotyped by flow cytometry and the percentage of MSCs in the sample was determined. Only samples with a MSC content of at least 98% were used. On the day of administration, the cells (25 million) were removed from the xeno-free substrate. No animal components were used and we substituted of animal trypsin which is the Trypsin recombinant (BioInd, Israel). The trypsin was inactivated with 1% albumin solution (Baxter)

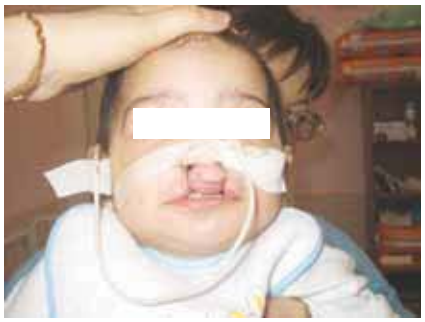

Fig. 1. Patient H., 6 months, diagnosis of congenital bilateral cleft of upper lip and palate

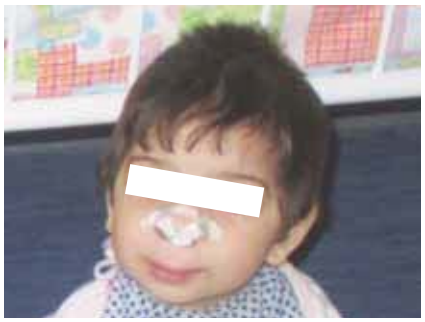

Fig. 2. Patient H., 2 years 6 months. After the first stage of surgical treatment by cheilorhinoplasty surgery

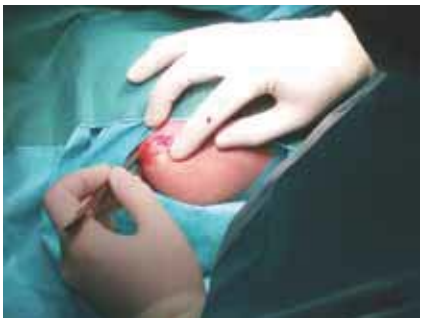

Fig. 3. The stage of harvesting adipose tissue from the gluteal fold

Fig. 4. Adipose tissue is placed in a container for transportation to the stem cell bank

Fig. 5. Impregnation of the osteogenic matrix with stem cells

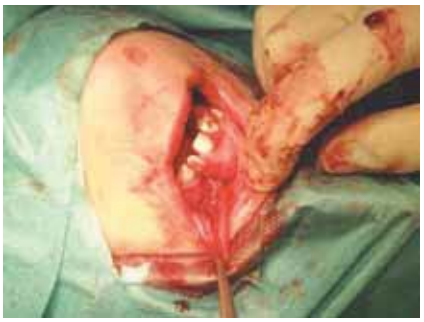

Fig. 6. The matrix impregnated with stem cells is placed in the defect region of the alveolar process of the upper jaw

in isotonic NaCl solution, washed by centrifugation and resuspended in 3 ml of isotonic NaCl solution.

When autologous MSCs were used, 3–4 weeks prior to the proposed surgery, 3 cm^3 of subcutaneous fat was harvested from the gluteal region of the patients (Figures 3, 4). The tissue was mechanically crushed, then incubated at 37°C in a 0.2% solution of collagenases (Type I, IV, Sigma-Aldrich, USA) in the PBS. The dissociated cells were washed from the enzyme by centrifugation (400 G, 10 min) and inoculated in vials at a density of 100–400 thousand cells/cm^2. The samples were cultured and analyzed in the same manner as described above for the allogeneic material.

In the case of autologous transplantations, a mixture of MSCs and preosteocytes was used (1 : 4.5 : 20 million). To obtain preosteocytes, an osteogenic medium containing no animal components (BioInd, Israel) was added to the MSC culture (80–90% confluency) and cultured without additional inoculations for 10 days. We replaced the medium every 3 days. The course of differentiation was monitored by PCR for the emergence of osteocalcin and cbfa1 in the mRNA samples and the control samples were stained for calcifications with 2% alizarin. On the day of transplantation, MSCs and preosteocytes were removed from the substrate, as described above, for allogenic material.

The "Biomatrix" membrane [decalcified material: 100% collagen and bone sulfated glycosaminoglycans (sGAG) of at least 1.5 mg/cm^3] was chosen as the osteogenic matrix that was filled with stem cells. It is a collagenic, completely resorbable material.

The osteogenic matrix was impregnated with mesenchymal stem cells and placed in the area of the bone defect (Figure 5, 6). Previously, using a bur, a compact plate of the end sections of the bone was removed in the region of fragments of the alveolar process of the upper jaw or in the region of the palatine processes of the upper jaw.

The control group of patients consisted of 40 people aged 2–4 years with the same pathology. Surgical treatment was conducted in a similar manner, but without the use of MSCs.

Discussion and results

The results of treatment were assessed on a three-point scale: good, satisfactory and unsatisfactory. The evaluation criteria were the presence of

postoperative complications, restoration of the function of the palato-pharyngeal ring, dissociation of the oral and nasal cavities, and radiographic signs of bone formation in the area of the upper jaw defect.

A good result was the absence of postoperative complications, restoration of the function of the palato-pharyngeal ring, dissociation of the oral and nasal cavity, and radiographic signs of the formation of bone tissue in the area of the defect of the upper jaw. Satisfactory results were determined by the absence of postoperative complications, restoration of the function of the palato-pharyngeal ring, and dissociation of the oral and nasal cavities. An unsatisfactory result was the formation of an orostoma and the absence of a closure of the palato-pharyngeal ring.

To assess the formation of bone tissue in the area of the diastasis, we used multispiral computed tomography (MSCT) of the skull. When using the method of computed tomography for the visual and quantitative determination of the density of structures, an X-ray attenuation scale, called the Hounsfield scale (black and white spectrum of the image) was used. The range of units of scale (densitometric indicators, English Hounsfield units) averages from –1024 to +1024. 0 HU is the average value in the Hounsfield scale and corresponds to the density of water. Negative scale values correspond to air and adipose tissues, while positive values correspond to soft tissues and denser bone tissues (Figure 7).

A good result was obtained in all 6 patients treated with MSCs. Complete dissociation of the oral and nasal cavity was achieved. The function of the palato-pharyngeal ring was restored. In treatment of pediatric patients with unilateral and bilateral cleft palate, the continuity of the alveolar process of the upper jaw was restored, and the fissure of the mouth vestibule was eliminated. At the follow up X-ray examination after the surgery in the area of the bone defect after the application of cellular technologies new tissue was found, corresponding to the bone tissue according to the density (Fig. 8, 9). The density on the Hounsfield scale ranged from 65 to 110 HU.

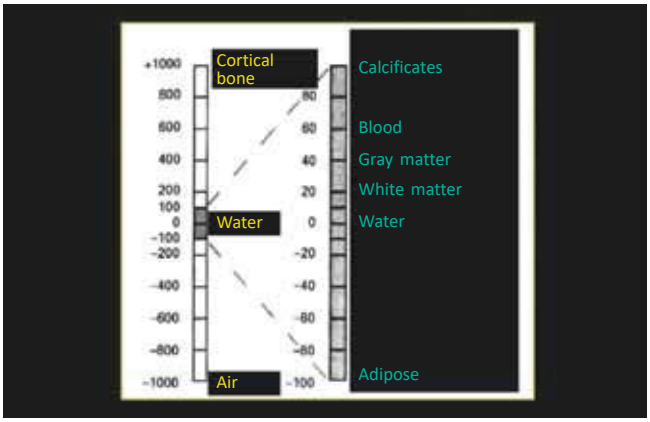

Fig. 7. The Hounsfield Scale

In the control group ($n = 40$), no bone formation was observed in the defect area in any of the cases. This enabled the treatment outcome to be considered satisfactory. Satisfactory results were obtained because the tissue did not approach the bone in the defect area (Fig. 10).

We evaluated both the immediate and long-term treatment results of pediatric patients with congenital cleft palates.

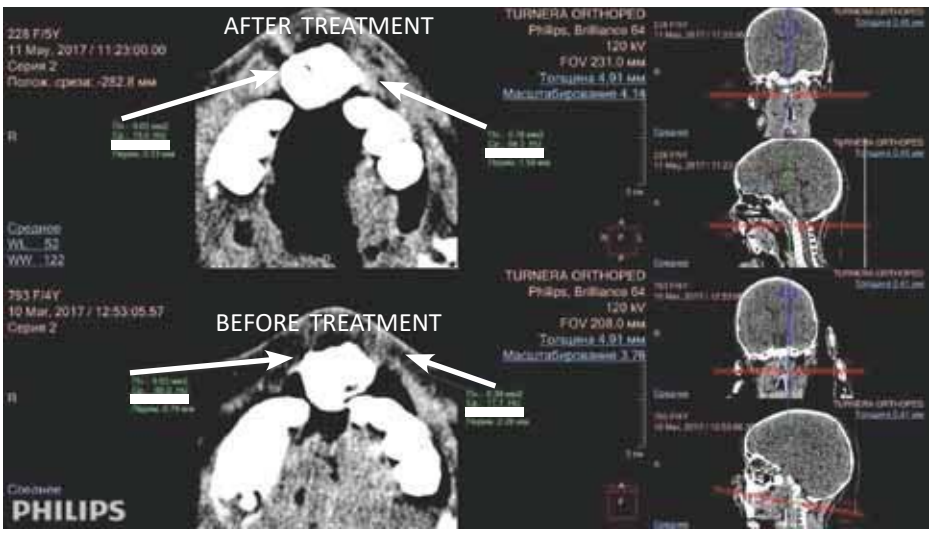

Fig. 8. Computed tomography of patients with bilateral clefts of the alveolar process of the upper jaw before and after treatment using mesenchymal stromal cells from both sides; after the treatment, the density indicates the formation of bone tissue

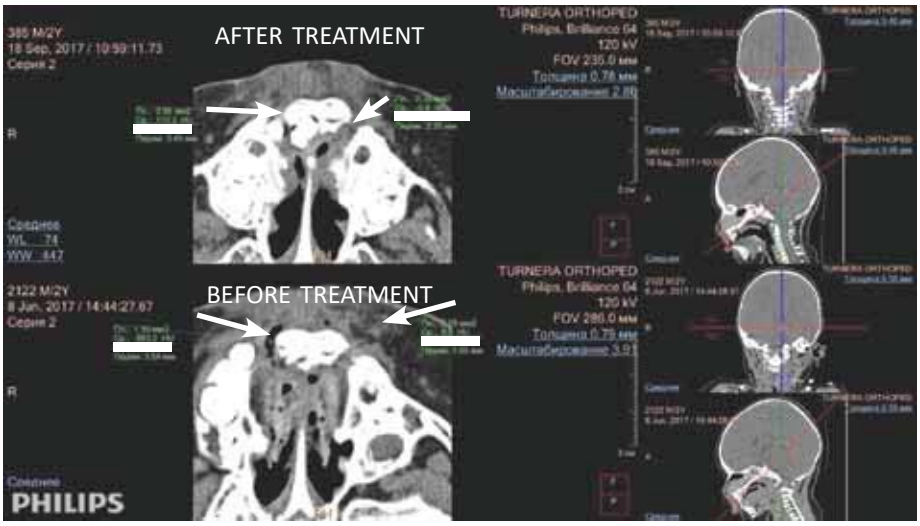

Fig. 9. Computed tomography of patients with bilateral clefts of the alveolar process of the upper jaw before and after treatment using mesenchymal stromal cells on the right only. On the left there is a defect between the cleaved sections of the alveolar process, and on the right, where mesenchymal stromal cells were applied, the defect is filled with bone tissue

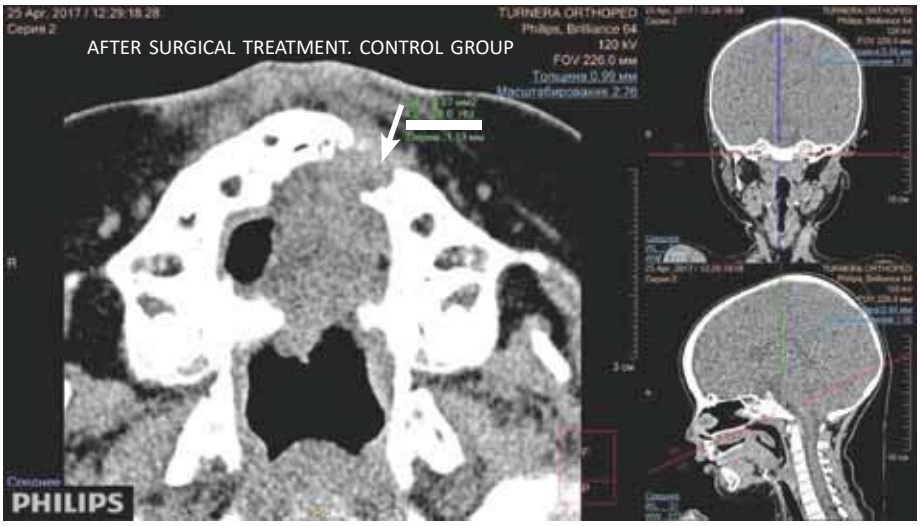

Fig. 10. Computed tomography of the patient from the control group after the surgery. Bone tissue is not formed

There were no significant differences in the timing of wound healing during the postoperative period.

Conclusion

Tissue engineering helps treat the congenital pathology of MFA. There are good prospects for using MSCs for the surgical treatment of extensive defects in the facial skeleton.

These procedures take advantage of high regenerative activity of transplanted stem cells due to stimulating effects on angiogenesis and the ability to differentiate into osteoblasts and chondroblasts to restore bone and cartilaginous tissues.

For reliable study of regenerative abilities of tissue engineering technology to restore bone defects it is necessary to perform preclinical and clinical studies under laboratory controls. Use of morphological research methods enable dynamic evaluation of the severity and orientation of regenerative processes.

Funding and conflict of interest

The work was supported by the Turner Scientific and Research Institute for Children's Orthopedics of the Ministry of Health of Russia as a part of scientific research project. The authors declare no obvious and potential conflicts of interest related to the publication of this article.

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